PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/53, 15/82, 9/02, A01H 5/00 — A2

(11) International Publication Number: WO 96/13591

(43) International Publication Date: 9 May 1996 (09.05.96)

(21) International Application Number: PCT/US95/13784

(22) International Filing Date: 25 October 1995 (25.10.95)

(30) Priority Data:
08/329,560 26 October 1994 (26.10.94) US

(71) Applicant: MICHIGAN STATE UNIVERSITY [US/US]; 238 Administration Building, East Lansing, MI 48824 (US).

(72) Inventors: CAHOON, Edgar, B.; Building 463, Brookhaven National Laboratories, Upton, NY 11973 (US). OHLROGGE, John, B.; 2123 Lagoon Drive, Okemos, MI 48864 (US).

(74) Agents: YATES, Michael, E. et al.; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).

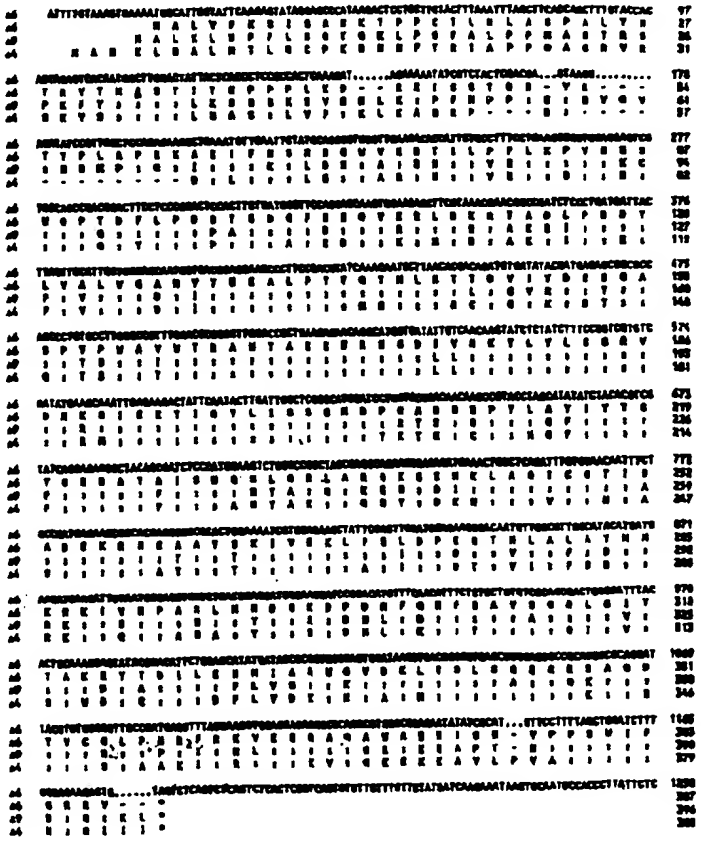
(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: METHODS AND COMPOSITIONS RELATING TO PLANT Δ^6 PALMITOYL-ACYL CARRIER PROTEIN DESATURASE

(57) Abstract

A plant Δ^6 palmitoyl-acyl carrier protein desaturase, the gene encoding the desaturase, and transgenic plants and plant cells containing the heterologous DNA encoding the desaturase are described. The desaturase introduces a double bond at the sixth carbon atom from the carboxyl end of a 16 carbon saturated fatty acid, and is therefore useful in production of plant seeds having a modified fatty acid profile.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

METHODS AND COMPOSITIONS RELATING TO PLANT Δ^6 PALMITOYL-ACYL CARRIER PROTEIN DESATURASE

Field of the Invention

The present invention relates to plant fatty acid desaturases. More particularly, the present invention relates to plant Δ^6 palmitoyl-acyl carrier protein desaturases, the genes encoding such desaturases, transgenic plants and plant cells containing DNA encoding such desaturases, and methods for altering the fatty acid profile of plant seeds through the use of such desaturases.

BACKGROUND

Fatty acid desaturases of plants have received considerable attention because of their contributions to the physiology and economic value of plants. The activity of fatty acid desaturases, for example, may be a component of the ability of certain species to adjust levels of membrane unsaturation in response to stresses such as chilling (1-3). In addition, the degree of fatty acid unsaturation resulting from desaturase activity is often a major determinant of the nutritional and industrial quality of plant seed oils (4).

Plants typically contain a variety of fatty acid desaturases. The most numerous of these are membrane-associated desaturases that use fatty acids bound to glycerolipids as substrates (5). In addition, the synthesis of oleic acid (18:1Δ^9) in plants and certain other organisms such as *Euglena* (photoauxotrophic) is catalyzed by a desaturase that functions on fatty acids esterified to acyl carrier protein (ACP) (6-8). This enzyme, the Δ^9 stearoyl (18:0)-ACP desaturase (EC 1.14.99.6), displays soluble activity in contrast to all previously characterized desaturases (6). In the presence of radiolabeled 18:0-ACP and cofactors including NADPH, ferredoxin (Fd), and ferredoxin-NADPH reductase, the activity of the Δ^9 18:0-ACP desaturase is readily detectable in extracts of most plant tissues (9). Due in part to its soluble nature and relative ease of assay, the $\Delta^9$18:0-ACP desaturase has been purified from several plant sources (10-14), and a number of cDNAs encoding

this enzyme have been isolated (12-20). In addition to the $\Delta^9$18:0-ACP desaturase, a $\Delta^4$16:0-ACP desaturase has recently been identified in plants (21, 22). This enzyme is a component of the petroselinic acid (18:1Δ^6) biosynthetic pathway in endosperm of coriander (*Coriandrum sativum* L.) and other Umbelliferae species. Translation of a cDNA for the $\Delta^4$16:0-ACP desaturase has revealed that this enzyme shares extensive amino acid identity with the $\Delta^9$18:0-ACP desaturase (21).

The existence of structurally related acyl-ACP desaturases with different substrate recognition and double bond-positioning properties offers the opportunity to compare the active site structures of members of this family of enzymes using techniques such as site-directed mutagenesis and x-ray crystallography. Information gained from this research could potentially lead to the design of desaturases capable of producing new industrially useful isomers of monounsaturated fatty acids. These studies would be aided by the isolation of cDNAs for other variant acyl-ACP desaturases in addition to those for the $\Delta^9$18:0-and $\Delta^4$16:0-ACP desaturases. A potential source of such a desaturase is seed of *Thunbergia alata* (Acanthaceae family). The oil of this tissue consists of more than 80% weight of the unusual fatty acid Δ^6 hexadecenoic acid (16:1Δ^6) (23). We have used biochemical and molecular biological approaches to examine whether 16:1Δ^6 is synthesized by the activity of a unique acyl-ACP desaturase that is related to the $\Delta^9$18:0- and $\Delta^4$16:0-ACP desaturases.

SUMMARY

The present invention therefore relates to an isolated Δ^6 palmitoyl acyl carrier protein desaturase and DNA encoding same, transgenic plants and plant cells containing heterologous DNA encoding said desaturase, mRNA derived from DNA encoding such a desaturase, and vectors containing DNA encoding the desaturase. The present invention also relates to a method for modifying the fatty acid content of a plant seed involving the introduction of a double bond at the sixth carbon atom from the carboxyl end of the saturated 16 carbon fatty acid, for example palmitic acid. Additionally, the present

invention relates to plant seeds having a modified fatty acid content derived through the method of the invention.

An objective of the invention is to provide a mechanism for the modification of fatty acids in order to enhance the industrial and nutritional quality of plant seed oils. As part of this invention, we have developed a method for introducing a double bond at a novel position in a saturated fatty acid. The invention entails the identification of the enzyme (a Δ^6- palmitoyl-acyl carrier protein desaturase) involved in the biosynthesis of the unusual fatty acid Δ^6-hexadecenoic acid. In addition, a complementary DNA (cDNA) was isolated for this desaturase. Expression of the cDNA in *Escherichia coli* resulted in the production of a catalytically active Δ^6-palmitoyl-acyl carrier protein desaturase.

This invention offers the opportunity to synthesize a monounsaturated fatty acid with a double bond positioned at the sixth carbon atom from the carboxyl end of the fatty acid molecule. The invention also allows for unsaturation to be introduced into a saturated 16 carbon fatty acid (palmitic acid). These two features of the invention allow for the production of a monounsaturated fatty acid that is not normally found in conventional plant seed oils. The major monounsaturated fatty acid present in seed oils is oleic acid, an 18 carbon fatty acid with a double bond positioned at the ninth carbon atom from the carboxyl end of the molecule.

The ability of the invention to introduce a double bond into palmitic acid offers the potential for reducing the saturated fatty acid content of vegetable oils. Typically, the presence of the 16 carbon saturated fatty acid palmitic acid limits the nutritional quality of seed oils. Therefore, the invention may allow for the production of a seed oil with reduced palmitic acid content. As a result, such an oil would presumably be less harmful to human health.

In addition, the product of the invention, Δ^6-hexadecenoic acid, may be useful as a chemical precursor of certain industrial feedstocks. Because the double bond of Δ^6-hexadecenoic acid is located at the sixth carbon atom, this fatty acid can be oxidatively cleaved to form adipic acid (a six carbon

dicarboxylic acid) and decanoic acid (a ten carbon fatty acid). Adipic acid is a precursor of nylon 66 and is currently derived from petroleum by-products.

Furthermore, the use of Δ^6-hexadecenoic acid has been proposed as an emollient in cosmetics (U.S. Patent No. 4,036,991).

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is an autoradiogram of intact products of [^{14}C]16:0-ACP desaturase assays conducted with a 100,000 x *g* supernatant of *T. alata* endosperm extracts (16:1), and the methyl ester of 16:0 derived from unreacted [1-^{14}C]16:0-ACP(16:0). The Std. lane contains methyl [1-^{14}C]16:0 (16:0), [1-^{14}C]18:1Δ^9(Δ^9), and [1-^{14}C]18:1Δ^6(Δ^6).

FIGURE 2 is an autoradiogram of oxidized products of [^{14}C]16:0-ACP desaturase assays. Shown are the permanganate-periodate oxidation products of the methyl ester derivatives of 16:1 formed by acyl-ACP desaturation activity in *T. alata* endosperm homogenates. [U-^{14}C]16:0-ACP was used as the substrate for this assay.

FIGURE 3 shows the time course of [1-^{14}C]16:0-ACP desaturase activity in a 100,000 x *g* supernatant of a *T. alata* endosperm homogenate. Assays were conducted with 118 pmol of [1-^{14}C]16:0-ACP and 23 µg of protein.

FIGURE 4 shows the effect of catalase, ferredoxin (Fd), nitrogen (N_2), potassium cyanide (KCN), or hydrogen peroxide (H_2O_2) on $\Delta^6$16:0-ACP desaturase activity in *T. alata* endosperm extracts. Assays were conducted for 10 min using 118 pmol of [1-^{14}C]16:0-ACP and 23 µg of protein from a 100,000 x *g* supernatant of endosperm homogenate. Assays with potassium cyanide and hydrogen peroxide contained 1mM of each compound, and catalase was omitted from assays containing hydrogen peroxide. (n.d.--not detected).

FIGURE 5 is the nucleotide sequence of the cDNA insert of pTAD4 (Δ^6) and a comparison of the deduced amino acid sequences of pTAD4 (Δ^5) and cDNAs for the coriander $\Delta^4$16:0-ACP desaturase (Δ^4) (see Ref. 21) and the castor $\Delta^9$18:0-ACP desaturase (Δ^9) (see Ref. 13). Identical amino acids are indicated by colons. Amino acids that are absent relative to the castor $\Delta^9$18:0-ACP desaturase are indicated by dashed lines. Alignment of the

nucleotide sequence of the cDNA insert of pTAD4 is maintained with a dotted line. The underlined alanine at amino acid 33 is the likely start of the native peptide encoded by pTAD4.

FIGURE 6 compares the $\Delta^5$16:0-ACP desaturase activity of extracts of *E. coli* BL21 pLysS containing only the vector pET3d or pET3d with insert derived from the *T. alata* cDNA of pTAD4 (with or without isopropyl-1-thio-β-D-galactopyranoside induction). The methyl ester of 16:1 formed by the *E. coli*-expressed desaturase was separated from methyl 16:0 of the unreacted substrate by argentation TLC as shown. Assays were conducted for 60 min using 230 μg of *E. coli* protein and 118 pmol of [1-^{14}C]16:0-ACP. Shown in the standard chromatogram are (std.) are methyl [^{14}C]16:0, -18:1Δ^9, and -18:1Δ^6.

FIGURE 7 shows substrate specificity and ferredoxin dependence of the *E. coli*-expressed *T. alata* $\Delta^6$16:0-ACP desaturase encoded by the cDNA insert of pTAD4. Assays were performed for 10 minutes using 118 pmol of either [1-^{14}C]14:0-, 16:0-(± ferredoxin, Fd), or 18:0-ACP and 65 μg of total *E. coli* protein.

FIGURE 8 shows a mass spectrum of derivatives of [1-^{14}C]16:1 formed by 16:0-ACP desaturase activity in extracts of *E. coli* expressing the mature peptide-coding region of pTAD4. The [1-^{14}C]16:1 desaturation product was converted to a methyl ester derivative and reacted with dimethyl sulfoxide prior to mass spectral analysis.

DETAILED DESCRIPTION

Experimental Procedures

Plant Material - Studies were conducted using developing endosperm dissected from fruits of *T. alata* Bojer ex Sims (black-eyed susan vine) (Northrup King, Minneapolis, MN). Fruits were collected from plants grown either outdoors in pots during summers in East Lansing, Michigan or under greenhouse conditions with natural illumination. In the latter case, flowers required hand pollination for adequate fruit set. Endosperm was frozen in liquid nitrogen following dissection and stored at -70°C until use in enzyme assays of RNA extraction.

Acyl-ACP Desaturation Assays - Approximately 200-300 mg of developing *T. alata* endosperm was homogenized in 3 ml of buffer consisting of 100 mM Tris-HCl, pH 7.5, 2.5 mM dithiothreitol, 1 mM isoascorbate, 10% (v/v) glycerol, and 1.5% (w/v) polyvinylpolypyrrolidone using an Elvehjem tissue grinder. Debris and polyvinylpolypyrrolidone were subsequently removed by centrifugation at 14,000 x *g* for 5 min. The supernatant was then passed through two layers of miracloth (Calbiochem) and spun for an additional 10 min at 30,000 x *g*. The soluble phase was removed while attempting to avoid recovery of the floating fat layer. A portion of contaminating fat was extracted by passing the supernatant through glass wool loosely packed in a Pasteur pipette. The supernatant from the 30,000 x *g* spin was further clarified by centrifugation at 100,000 x *g* for 60 min. All centrifugation steps were performed at 5°C. The resulting supernatant was used immediately for desaturation assays described below or frozen in aliquots in liquid N_2 and stored at -70°C until further use. Of note, extracts developed a brown color, presumably due to extensive phenolic oxidation, when maintained at -20°C for longer than 1-2 weeks.

Acyl-ACP desaturation assays were based on those previously described (8, 9). Assays were performed in a total volume of 150 µl in loosely capped 13 x 100-mm glass tubes and consisted of 1.25 mM NADPH (from a freshly prepared stock in 100 mM Tricine, pH 8.2), 3.3 mM ascorbate, 0.7 mM dithiothreitol, 8000 units of bovine liver catalase (Sigma), 5 µg of bovine serum albumin (Fraction V) (Sigma), 20 µg of spinach ferredoxin (Sigma), 80 milliunits of spinach ferredoxin:NADPH reductase (Sigma), 33 mM PIPES, pH 6.0, and 118 pmol of [1-^{14}C] acyl-ACP or -CoA. Reactions were started with the addition of the 100,000 x *g* supernatant of homogenized *T. alata* endosperm (typically 20-25 µg of total protein) and were conducted at room temperature (~22°C) with shaking (100 revolutions/min). Assays were terminated with the addition of 850 µl of 2.35 M NaOH and carrier fatty acids (30 µg of palmitic and petroselinic acid). The stopped reactions were then heated at 85°C for 1 hour. Following acidification with 350 µl of 4M H_2SO_4, the resulting free fatty acids were recovered by three extractions with 2.5 ml

of hexane. Fatty acids were converted to methyl ester derivatives with 10% (w/v) boron trichloride in methanol (Alltech) using the method described by Morrison and Smith (24). Reaction products were then analyzed on 15% $AgNO_3$ TLC plates developed sequentially to heights of 10 and 20 cm in toluene at -20°C. TLC plates were prepared as described previously (25). Radioactivity was detected by autoradiography and quantified by liquid scintillation counting of TLC scrapings in a non-aqueous complete mixture.

To confirm the identity of $16:1\Delta^6$ produced from palmitoyl-ACP, assays were conducted as described above using [U-^{14}C] palmitoyl-ACP as the substrate. The methyl ester derivative of the monounsaturated product was purified by argentation TLC as described above and eluted from TLC scrapings with hexane/ethyl ether (2:1, v/v). The monounsaturated methyl ester was then cleaved at its double bond using permanganate-periodate oxidation (26). Chain lengths of oxidation products were determined relative to [^{14}C] fatty acid standards by reverse-phase TLC using a mobile phase of acetonitrile/methanol/water (75:25:0.5).

Inhibition of desaturase activity was examined by supplementing assays with 1 mM KCN (neutralized) or 1 mM H_2O_2. In the latter case, catalase was omitted from reactions. Oxygen dependence of desaturase activity was characterized by purging assay tubes completely with nitrogen prior to and after addition of plant extract, and the reaction tube was tightly capped for the duration of the assay.

Radiolabeled acyl-ACPs were synthesized enzymatically using *Escherichia coli* ACP according to the method of Rock and Garwin (27). The following fatty acids were used in the synthesis of acyl-ACPs: [1-^{14}C] myristic acid (American Radiolabeled Chemicals, St. Louis, MO) (specific activity 55 mCi/mmol), [1-^{14}C] palmitic acid (NEN Dupont) (specific activity 58 mCi/mmol), [U-^{14}C] palmitic acid (NEN Dupont) (specific activity 800 mCi/mmol), and [1-^{14}C] stearic acid (American Radiolabeled Chemicals) (specific activity 55 mCi/mmol). [1-^{14}C] Palmitoyl-CoA (specific activity 52 mCi/mmol) was purchased from Amersham Corp. A [^{14}C] petroselinic acid

standard was prepared by incubation of coriander endosperm slices in [1-^{14}C] acetate as described previously (25).

T. alata Endosperm cDNA Library Construction - Total RNA was isolated from *T. alata* endosperm using the method of Hall et al. (28). RNA was then passed through a cellulose (Sigma Cell 50, Sigma) column in order to reduce amounts of polysaccharides potentially recovered along with the RNA.

Poly(A)$^+$ RNA was enriched by passing total RNA once through a column of oligo (dT) cellulose (Pharmacia LKB Biotechnology Inc.) and subsequently used in the construction of a Uni-ZAP XR (Stratagene) cDNA expression library according to the instructions of the manufacturer. A portion of the total amplified library packaged in phage was mass excised (29) yielding pBluescript II SK(-) harboring cDNA inserts. The recovered plasmid DNA was used for cDNA isolation by colony hybridization and polymerase chain reaction (PCR) amplification as described below.

PCR Amplification of Nucleotide Sequences Encoding Acyl-ACP Desaturases - Fully degenerate sense and antisense oligonucleotides were prepared that corresponded respectively to the conserved amino acid sequences Gly-Asp-Met-Ile-Thr-Glu-Glu and Glu-Lys-Thr-Ile-Gln-Tyr-Leu present in $\Delta^9$18:0 - (13, 15-20) and $\Delta^4$16:0-ACP desaturases (21). The sequence of the resulting sense and antisense oligonucleotides were 5'-GG(A/C/G/T)GA(C/T)ATGAT(A/C/T)AC(A/C/G/T)GA(A/G)GA-3' and 5'-A(A/G)(A/G)TATTG(A/G/T)AT(A/C/G/T)GT(C/T)TT(C/T)TC-3', respectively. Included on the 5' terminus of each oligonucleotide was sequence (5'-CAUCAUCAUCAU-3' or 5'-CUACUACUACUA-3') that allowed for insertion of PCR products into the pAMP1 vector (Life Technologies, Inc.). Template for PCR amplification was generated by transformation of the SOLR strain (Stratagene) of *E. coli* with an aliquot of the mass-excised *T. alata* endosperm cDNA library. Following growth of transformed *E. coli* to stationary phase in 3 ml of liquid culture, plasmid DNA was purified for use as template in PCR amplification. Reactions were performed in a 50-µl volume and consisted of 10 µM sense and antisense oligonucleotides, 150-300 ng of plasmid DNA

derived from the *T. alata* cDNA library, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 x *Taq* reaction buffer (Life Technologies, Inc.), and 5 units of *Taq* polymerase (Life Technologies, Inc.). Temperature conditions for PCR amplification were 5 min at 95°C and 25 cycles of 1 min at 95°C, 1.5 min at 55°C, and 1.5 min at 72°C. This was followed by an additional 10 min extension at 72°C. PCR fragments of approximately 215 base pairs were gel-purified, ligated into the pAMP1 vector using the CloneAmp system (Life Technologies, Inc.) according to the manufacturer's protocol, and introduced into *E. coli* DH5*a* (Life Technologies, Inc.). The resulting colonies were screened using colony hybridization as described by Sambrook et al. (30). A "negative" screening protocol was used to reduce the chances of reisolating cDNAs (pTAD1, 2, and 3) encoding $\Delta^9$18:0-ACP desaturases that were previously obtained by antibody screening of the *T. alata* endosperm cDNA library (19). DNA probes for library screening were formed by PCR amplification of portions of pTAD 1,2, and 3. Primers and PCR reaction conditions were the same as those described above. An equimolar mixture of the PCR products derived from pTAD1, 2, and 3 was used as template for the synthesis of [α-^{32}P]dCTP random-primed labeled probes. Hybridization of plasmids of lysed colonies with radiolabeled probes was carried out in 6 x SSC and 0.25% (w/v) non-fat dry milk with shaking for 4 hours at 53°C as described by Sambrook et al. (30). Filters were washed three times in 1 x SSC and 0.1% SDS at 60°C (45 min/wash) and exposed to autoradiography. Plasmid DNA was subsequently isolated from 10 colonies which displayed little or no hybridization to the probes. Nucleotide sequence of the inserts of these plasmids was obtained by dideoxy chain termination using Sequenase 2.0 (United States Biochemical Inc.) according to the manufacturer's instructions. Two classes of plasmids were identified (designated pEC6 and 7), both of which contained inserts encoding portions of apparent acyl-ACP desaturases (based on amino acid identity with known $\Delta^9$18:0- and $\Delta^4$16:0-ACP desaturases).

Screening of a T. alata Endosperm cDNA Library for a Full-length Divergent Acyl-ACP Desaturase - Aliquots of the mass excised *T. alata* endosperm cDNA library were used to transform *E. coli* SOLR cells.

Approximately 50,000 colonies were screened using colony hybridization as described previously (30). Nucleotide probes for screening were generated by [α-^{32}P]dCTP random-primed hexamer labeling of inserts of pEC6 and 7. Hybridization and washing conditions were the same as those described above. Colonies containing plasmid DNA that strongly hybridized to the probe derived from pEC6 were isolated, and nucleotide sequence was obtained from both strands of the longest cDNA insert (the corresponding plasmid was designated pTAD4) using Sequenase 2.0. Because of a relative lack of abundance, colonies containing plasmid hybridizing to the pEC7-derived probe were not further characterized.

E. coli Expression of a Putative cDNA for Δ^6 Palmitoyl-ACP Desaturase - To determine the activity of the desaturase encoded by pTAD4, the portion of the clone corresponding to the mature peptide (total protein minus plastid transit peptide) was expressed in *E. coli*. This region of the cDNA insert of pTAD4 was first amplified by PCR using Vent DNA polymerase (New England Biolabs). The nucleotide sequence of the sense primer was 5'-GCTTCGACTATTACTCAC3-'. M-13(-20) forward primer was used as the antisense oligonucleotide. The PCR product was blunt-end ligated into the *Nco*I site of the *E. coli* expression vector pET3d (Novagen) as described (30). The *Nco*I-digested vector had been previously treated with the Klenow fragment of DNA polymerase I to fill-in 5' protruding ends. The junction between the vector and the 5' terminus of the insert was sequenced to confirm that the PCR product was ligated into pET3d in the proper reading frame. This construct was subsequently introduced into the *E. coli* strain BL21 pLysS and grown in LB media with carbenicillin (125 μg/ml) and chloroamphenicol (30 μg/ml) selection. At a cell density of OD_{600} ~ 0.8, cultures were induced with the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.5 mM and grown for an additional 4 hours. Cells were then washed in 50 mM Tris-HC1, pH 7.5, lysed by two freeze-thaw cycles (using a liquid nitrogen bath for freezing and a 22°C water bath for thawing). Lysates were then incubated with bovine pancreas DNase I (Boehringer Mannheim) (20 μg/ml) for 15 min at 22°C. The

extract was subsequently centrifuged at 14,000 x *g* for 5 min. The resulting supernatant was used for acyl-ACP desaturation assays as described above. Radiolabel in the TLC-analyzed reaction products was detected using a Bioscan System 200 image scanner. The double bond position of the monounsaturated product was determined by gas chromatography-mass spectrometry analysis of its dimethyl disulfide derivative (31). In these studies, the desaturation assays described above were scaled up 6-fold, and reactions were conducted with 2.6 nmol of [1-^{14}C] 16:0-ACP and 1.1 mg of soluble protein of lysed *E. coli* expressing the *T. alata* cDNA. Assays were conducted for 4 hours. High protein concentrations and long incubation periods were used to ensure the synthesis of sufficient amounts of monounsaturated fatty acid for mass spectral analyses. Reaction products were converted to fatty acid methyl esters as described above and subsequently reacted with 100 µl of an iodine solution (60 mg/ml ethyl ether) and 350 µl of dimethyl disulfide (Aldrich). After 3 hours of incubation with shaking (250 revolutions/min) at 37°C, dimethyl disulfide derivatives of unsaturated fatty acid methyl esters were extracted as described previously (32). These derivatives (dissolved in hexane) were then analyzed by gas chromatography-mass spectrometry using a Hewlett Packard PH5890II gas chromatograph interfaced with a HP5971 mass selective detector. Separation of analytes was achieved using a DB23 (30 m x 0.25 mm inner diameter) column (J&W Scientific) with the oven temperature programmed from 185°C (3 min hold) to 230°C at rate of 2.5°C/min.

Results

Detection of a Soluble Δ^6 Palmitoyl-ACP Desaturase in T. alata Endosperm Extracts - The seed oil of *T. alata* is composed of nearly 85% weight of the unusual monounsaturated fatty acid Δ^6 hexadecenoic acid (16:1Δ^6). To examine the metabolic origin of the double bond of this fatty acid, the 100,000 x *g* supernatant of a homogenate of developing *T. alata* seed endosperm was incubated with [^{14}C]16:0-ACP and potential desaturase cofactors. Using this assay system, substantial amounts of 16:0-ACP desaturase activity were detected in the soluble endosperm extract (Fig. 1).

In the absence of a radiolabeled standard for 16:1Δ^6, two independent analytical methods indicated that the double bond of the resulting 16:1 moiety was positioned at the Δ^6 carbon atom: 1) the 16:1 desaturation product displayed mobility on argentation TLC plates similar to that of the Δ^6 monounsaturated fatty acid petroselinic acid (18:1Δ^6) when these molecules were analyzed as methyl ester derivatives (Fig. 1) and 2) permanganate-periodate oxidation of the methyl ester of the 16:1 desaturation product gave rise to a molecule with mobility on reverse-phase TLC equivalent to that of decanoic acid (10:0) (Fig. 2, *Product B*) as well as to an acyl moiety containing a lesser number of carbon atoms (Fig. 2, *Product A*).

Substrate Properties of the Δ^6 Acyl-ACP Desaturase - To confirm that the Δ^6 desaturase identified above is most active with 16:0-ACP, assays were conducted using ^{14}C-saturated acyl-ACP substrates containing 14, 16, and 18 carbons. As with 16:0-ACP (described above), Δ^6 desaturase activity was also detected when [1-^{14}C]14:0- and 18:0-ACP were reacted with a 100,000 x *g* supernatant of a *T. alata* endosperm homogenate. Following derivitization, a portion of the desaturation products resulting from 18:0-ACP comigrated on argentation TLC with the methyl ester of petroselinic acid, which was resolvable in this system from methyl oleic acid (data now shown). Similarly, the desaturation product arising from 14:0-ACP migrated in the expected position for a fatty acid containing a Δ^6 double bond (data not shown). Therefore, it appears that the Δ^6 desaturase of *T. alata* endosperm positions the placement of unsaturation with regard to the carboxyl end of fatty acid substrates. This double bond positioning property has been previously observed with the $\Delta^9$18:0- and $\Delta^4$16:0-ACP desaturases (22, 33).

Under the assay conditions used, $\Delta^6$16:0-ACP desaturase activity in the 100,000 x *g* supernatant of a *T. alata* endosperm homogenate was essentially linear over 10 min (Fig. 3). When assays were conducted over this time period, the specific activity of the Δ^6 desaturase was approximately 7-fold higher using [1-^{14}C]16:0-ACP as a substrate rather than either [1-^{14}C]14:0- or 18:0-ACP (Table 1). Values obtained with the latter substrate, however, were obscured because of the presence of completing $\Delta^9$18:0-ACP

desaturase activity in the endosperm extract. Finally, no desaturase activity was detected when [1-^{14}C]16:0-CoA was presented as a potential substrate. Overall, these results indicate that the Δ^6 desaturase is most active *in vitro* with 16:0 esterified to ACP.

TABLE I

*In vitro substrate specificities of acyl-*ACP or -CoA *desaturases of T. alata endosperm*

Desaturase assays were conducted for 10 min using 118 pmol of [1-^{14}C]acyl-ACP or -CoA substrate and 23 µg of total protein from 100,000 x *g* supernatant of a *T. alata* endosperm homogenate.

	Monounsaturated products[a]	
Substrate	Δ^6	Δ^9
	pmol/min/mg protein	
14:0-ACP	13	ND[b]
16:0-ACP	99	ND
16:0-CoA	ND	ND
18:0-ACP	12	173[c]

[a]14:1Δ^6, 18:1Δ^6, and 18:1Δ^9 were identified by the mobilities of these fatty acids on argentation TLC plates.

[b]Not detected.

[c]Assay conditions were adjusted only for the linear measurement of Δ^6 desaturase activity. Therefore this value may underestimate the specific activity of $\Delta^9$18:0-ACP desaturase.

Cofactors and Inhibitors of Δ^6 Palmitoyl-ACP Desaturase Activity - Additional *in vitro* assays were conducted to compare the functional properties of the $\Delta^6$16:0-ACP desaturase with those previously determined for the $\Delta^9$18:0-ACP desaturase (7, 8, 10). In this regard, virtually no $\Delta^6$16:0-ACP desaturase activity was detected in the 100,000 x *g* supernatant of *T. alata* endosperm homogenates when assays were conducted in the absence of ferredoxin or molecular oxygen (Fig. 4). $\Delta^6$16:0-ACP desaturase activity was also reduced when catalase was omitted from assays. Furthermore, the inclusion of 1 mM KCN or H_2O_2 in reactions resulted in the loss of most of the desaturase activity. Such catalytic properties of the *T. alata* $\Delta^6$16:0-ACP desaturase were similar to those previously described for the $\Delta^9$18:0-ACP desaturase (7, 8, 10).

Isolation of a cDNA Encoding a Diverged Acyl-ACP Desaturase from T. alata Endosperm - Based on functional similarities of the $\Delta^6$16:0 - and $\Delta^9$18:0-ACP desaturases described above, we examined whether these enzymes are also structurally related. To address this question, attempts were made to isolate a cDNA for the $\Delta^6$16:0-ACP desaturase using $\Delta^9$18:0-ACP desaturase-derived probes. As a first approach, a cDNA expression library prepared from poly(A)$^+$ RNA of *T. alata* endosperm was screened with antibodies against the $\Delta^9$18:0-ACP desaturase of avocado (13). This method was previously used to obtain a cDNA for the $\Delta^4$16:0-ACP desaturase of coriander endosperm (21). In the present study, however, antibody screening of the *T. alata* endosperm expression library yielded only cDNAs for three apparent isoforms of the $\Delta^9$18:0-ACP desaturase, which were designated pTAD1, 2, and 3 (19).

As an alternative approach, PCR amplification of a $\Delta^6$16:0-ACP desaturase-specific nucleotide probe was attempted using degenerate sense and antisense oligonucleotides prepared against two conserved amino acid sequences in $\Delta^9$18:0- and $\Delta^4$16:0-ACP desaturases. One of the sequences (Gly-Asp-Met-Ile-Thr-Glu-Glu) is encoded by the cDNA for the $\Delta^4$16:0-ACP desaturase and all known cDNAs for the $\Delta^9$18:0-ACP desaturase. The second sequence (Glu-Lys-Thr-Ile-Gln-Tyr-Leu) is also encoded by the

$\Delta^4$16:0-ACP desaturase of cDNA and all known $\Delta^9$18:0-ACP desaturase cDNAs except that of safflower (14). Products of approximately 215 base pairs obtained following one round of PCR amplification of the total *T. alata* cDNA library (in plasmid form) were screened after subcloning into the pAMP1 vector. To delineate products of the previously isolated cDNAs pTAD1, 2, and 3, colonies containing PCR-derived clones were screened in a negative manner with random-labeled probes for pTAD1, 2, and 3 and conditions of moderate to high stringency. One of the resulting clones (pEC6) that displayed weak or no hybridization to these probes encoded an amino acid sequence that was somewhat diverged from those of known $\Delta^9$18:0-ACP desaturases.

When the *T. alata* endosperm library was screened with a random-labeled probe prepared from the insert of pEC6, ≥0.1% of the total cDNAs examined strongly hybridized to this probe. The longest of a selected portion of these cDNAs (the corresponding plasmid was designated pTAD4) contained 1279 base pairs and had an open-reading frame corresponding to a 387-amino-acid polypeptide with considerable identity to known $\Delta^4$16:0- and $\Delta^9$18:0-ACP desaturases (Fig. 5). Based on similarity of flanking bases to the consensus sequence proposed by Lütcke et al. (34), the translational start site of the cDNA insert of pTAD4 likely occurs at nucleotide 17. In addition, from homology with $\Delta^4$16:0- and $\Delta^9$18:0-ACP desaturases, the mature peptide encoded by pTAD4 likely begins at amino acid 33. As such, the 32 amino acids preceding this residue correspond to a putative plastid transit peptide as is present in all acyl-ACP desaturases characterized to date.

Interestingly, the cDNA insert of pTAD4 lacks nucleotide sequence for 6-7 amino acids found near the amino terminus of all previously characterized $\Delta^9$18:0-ACP desaturases. This region is also altered in the cDNA for the coriander $\Delta^4$16:0-ACP desaturase (21) as compared to cDNAs for $\Delta^9$18:0-ACP desaturases. In this case, the coding sequence for 15 amino acids is absent in the $\Delta^4$16:0-ACP desaturase cDNA relative to the castor $\Delta^9$18:0-ACP desaturase cDNA (13) (Fig. 5). The pTAD4-encoded peptide also contains 2 less amino acids at its carboxyl terminus than both the $\Delta^4$16:0- and $\Delta^9$18:0-

ACP desaturases. Despite these differences, the interior regions of the putative desaturase encoded by pTAD4 share significant identity with portions of the primary structures of $\Delta^4$16:0- and $\Delta^9$18:0-ACP desaturases, and the spacing between conserved regions of amino acids is the same in all three desaturase types. Overall, the mature peptide encoded by the cDNA insert of pTAD4 shares 66% identity with the castor $\Delta^9$18:0-ACP desaturase and 57% identity with the coriander $\Delta^4$16:0-ACP desaturase, disregarding any missing amino acids.

Activity of an E. coli-expressed cDNA for a Diverged Acyl-ACP Desaturase of T. alata Endosperm - To determine the activity of the desaturase corresponding to the cDNA insert of pTAD4, the mature peptide-encoding region of this clone was expressed in *E. coli* with expression driven by the T7 RNA polymerase promoter of the vector pET3d (Novagen). When assayed with [1-^{14}C]16:0-ACP, crude extracts of isopropyl-1-thio-β-D-galacto-pyranoside-induced recombinant *E. coli* catalyzed the synthesis of [^{14}C]16:1 (Fig. 6). In addition, the methyl ester of the 16:1 product displayed mobility on argentation TLC plates similar to that of a methyl petroselinic acid (18:1Δ^6) standard, suggesting that this monounsaturated product is a Δ^6 isomer. Detectable acyl-ACP desaturase activity was absent in extracts of *E. coli* harboring the pET3d vector without cDNA insert or in uninducted recombinant *E. coli*. Furthermore, like the activity found in *T. alata* endosperm extracts, the desaturase expressed in *E. coli* displayed an *in vitro* substrate preference for 16:0-ACP and exhibited no detectable activity in the absence of reduced ferredoxin (Fig. 7).

The [^{14}C]16:0 moiety produced *in vitro* from the *E. coli*-expressed desaturase was conclusively identified as a Δ^6 isomer through gas chromatography-mass spectrometry analysis of its dimethyl disulfide derivative (Fig. 8). In the mass spectrum shown, the ions 145, 177, 187, and 364 *m/z* are diagnostic for a [1-^{14}C]16:1Δ^6 moiety. Significant amounts of non-radiolabeled or [^{12}C]16:1Δ^6 were also detected among the desaturase assay products. This was indicated by the presence of the additional ions 143, 175, and 362 *m/z* in the mass spectrum of [1-^{14}C]16:1Δ^6 as well as by an

enrichment in the abundance of ions 187 *m/z* (Fig. 8). It is unlikely that the non-radiolabeled 16:1Δ^6 resulted from *in vivo* synthesis in *E. coli*. In this regard, *E. coli* does not normally produce 16:1Δ^6 (35). Furthermore, gas chromatographic analysis of fatty acids of *E. coli* expressing the pTAD4-encoded desaturase failed to detect any 16:1Δ^6 in the bacterial lipids (data now shown). Given the relatively high concentrations of *E. coli* protein used in these assays, unlabeled 16:1Δ^6 likely arose from the *in vitro* desaturation of endogenous *E. coli* 16:0-ACP present in crude bacterial extracts.

Of note, expression levels of the *T. alata* cDNA in *E. coli* appeared to be low relative to that often obtained with DNA inserts placed behind the T7 RNA polymerase promoter (36). The expressed protein, for example, could not be distinguished on Coomassie-stained SDS-polyacrylamide gels of either the total soluble or insoluble protein fractions of lysed *E. coli* (data not shown). Also suggestive of low expression levels in *E. coli*, the specific activity of $\Delta^6$16:0-ACP desaturase in recombinant *E. coli* extracts (Fig. 7) was typically half of that detected in *T. alata* endosperm homogenates (Table I).

The results presented here demonstrate the involvement of a novel soluble $\Delta^6$16:0-ACP desaturase in the synthesis of Δ^6 hexadecenoic acid in the endosperm of *T. alata*. The activity of this enzyme has several properties similar to those previously described for the $\Delta^9$18:0-ACP desaturase. These include the requirement of reduced ferredoxin for detectable *in vitro* activity, the stimulation of activity by catalase, and the inhibition of activity by potassium cyanide and hydrogen peroxide. The existence of a $\Delta^6$16:0-ACP desaturase in *T. alata* endosperm was confirmed by the isolation of a cDNA for this enzyme. While the amino acid sequence deduced from this cDNA shares some identity with $\Delta^9$18:0- and $\Delta^4$16:0-ACP desaturases, these findings, together with those previously obtained for petroselinic acid biosynthesis (19, 21), indicate that natural variations in the primary structures of acyl-ACP desaturases can give rise to novel enzymes with altered substrate recognition and double bond positioning properties.

The major difference between the primary structures of the mature $\Delta^6$16:0-, $\Delta^4$16:0-, and $\Delta^9$18:0-ACP desaturases occurs in a region near their

amino termini. In this region, the *T. alata* $\Delta^6$16:0-ACP desaturase contains 6 less amino acids than the castor $\Delta^9$18:0-ACP desaturase. Similarly, this portion of the coriander $\Delta^4$16:0-ACP desaturase lacks 15 amino acids relative to the castor $\Delta^9$18:0-ACP desaturase. Without intending to be limited to any particular theory, one possibility is that differences in recognition of substrate chain length (16:0-ACP *versus* 18:0-ACP) and/or double bond positioning of these desaturases are associated with this divergence in the primary structures of these enzymes. Alternatively, this region of the amino terminus of $\Delta^9$18:0-ACP desaturase may not contribute significantly to the catalytic properties of this enzyme. As such, while again not intending to be limited to any particular theory, if the Δ^4 and $\Delta^6$16:0-ACP desaturases evolved from the $\Delta^9$18:0-ACP desaturase, then there may have been little selective pressure to maintain this region intact in the variant 16:0-ACP desaturases.

Ultimately, an understanding of how differences in the amino acid sequences of $\Delta^4$16:0-, $\Delta^6$16:0-, and $\Delta^9$18:0-ACP desaturases contribute to variations in their functional properties will require comparisons of the three-dimensional structures of these enzymes. In this regard, elucidation of the crystal structure of the castor $\Delta^9$18:0-ACP desaturase is currently in progress (37). With such information, it will be possible to overlap amino acid sequences of the Δ^4 and $\Delta^6$16:0-ACP desaturases onto the three-dimensional structure of $\Delta^9$18:0-ACP desaturase to more precisely identify residues associated with the different substrate recognition and double bond positioning properties of these enzymes. This could eventually lead to the design of "tailor-made" desaturases that are capable of inserting double bonds into a variety of positions of acyl moieties of a range of carbon chain lengths.

An interesting observation from the studies described above was the lack of detectable amounts of 16:1Δ^6 in lipids of *E. coli* expressing the *T. alata* cDNA. Similarly, Thompson et al. (14) reported that expression of the safflower $\Delta^9$18:0-ACP desaturase cDNA did not lead to the *in vivo* production of oleic acid in recombinant *E. coli*. The latter result can be explained by the fact that *E. coli* contains little 18:0-ACP (38). However, 16:0-ACP is a major

component of the acyl-ACP pool of *E. coli*. Therefore it is unlikely that the lack of 16:1Δ^6 synthesis in *E. coli* expressing the *T. alata* cDNA is due to the presence of insufficient substrate for the desaturase. In addition, *E. coli* has been reported to contain ferredoxin (39), the apparent electron donor for the $\Delta^6$16:0-ACP desaturase. However, as proposed by Thompson et al. (14), *E. coli* ferredoxin may not functionally interact with plant acyl-ACP desaturases. Alternatively, *E. coli* may not have adequate amounts of ferredoxin in a reduced form as required for $\Delta^6$16:0-ACP desaturase activity.

In addition to 16:1Δ^6, *T. alata* seed contains the unusual fatty acid 18:1Δ^8, which composes about 2% weight of the oil of this tissue (23). We have previously shown that petroselinic acid (18:1Δ^6) is formed by elongation of 16:1Δ^4-ACP in Umbelliferae endosperm (22). In an analogous manner, we predict that 18:1Δ^8 arises from the elongation of 16:1Δ^6-ACP rather than from the Δ^8 desaturation of 18:0-ACP. Unlike the synthesis of petroselinic acid, though, elongation of 16:1-ACP in *T. alata* endosperm is likely not a major pathway as the ratio of amounts of 16:1Δ^6:18:1Δ^8 in this tissue is approximately 40:1. In contrast, the ratio of amounts of 16:1Δ^4:18:1Δ^6 in endosperm of the Umbellifarae coriander is more than 1:500 (22, 25).

Finally, significant efforts have been directed toward the development of transgenic crops that produce high value specialty oils (4, 40, 41). Using methodologies currently well known in the art, transgenic plants could be produced (42, 43) which would contain and express the $\Delta^6$16:0-ACP desaturase gene and which would produce high levels of 16:1Δ^6. In this regard, oils rich in 16:1Δ^6 may have properties suitable for industrial use. Like petroselinic acid, 16:1Δ^6 can be oxidatively cleaved at its double bond to yield adipic acid, a precursor of nylon 6,6. In addition, high palmitic acid (16:0) mutants of crop plants including soybean (44) and *Brassica campesteris* (45) are available that could serve as appropriate backgrounds for transgenic expression of the cDNA for the *T. alata* $\Delta^6$16:0-ACP desaturase. Still the success of such research would likely require additional studies to determine whether enzymes other than $\Delta^6$16:0-ACP desaturase are

specialized for the synthesis and metabolism of 16:1Δ^6 in *T. alata* endosperm. For example, a petroselinoyl-ACP-specific thioesterase has been identified in Umbelliferae endosperm extracts that efficiently releases petroselinic acid from ACP and, as a result, makes this fatty acid available for subsequent storage in triacylglycerol (46). A related enzyme may also be required for high levels of 16:1Δ^6 accumulation in transgenic plants.

References

1. Cheesbrough, T. M. (1990) *Plant Physiol.* **93**. 555-559.
2. Wada, H., Gombos, Z., and Murata, N. (1990) *Nature* **347**. 200-203
3. Miquel, M., James Jr., Dr. Dooner, H., and Browse, J. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**. 6208-6212.
4. Battey, J. F., Schmid, K. M., and Ohlrogge, J. B. (1989) *Trends Biotech.* **7**. 122-125.
5. Heinz, E. (1993) in *Lipid Metabolism in Plants* (Moore, T. S., ed) pp. 33-89. CRC Press, Boca Raton, FL.
6. Nagai, J., and Bloch, K. (1965) *J. Biol. Chem.* **240**, 3702-3703.
7. Nagai, J., and Bloch, K. (1968) *J. Biol. Chem.* **243**. 4626-4633.
8. Jaworski, J. G., and Stumpf, P. K. (1974) *Arch. Bochem. Biophys.* **162**. 158-165.
9. McKeon, T. A., and Stumpf, P. K. (1981) *Methods Enzymol.* **71**. 275-281.
10. McKeon, T. A., and Stumpf, P. K. (1982) *J. Biol. Chem.* **257**. 12141-12147.
11. Cheesbrough, T. M., and Cho, S. H. (1990) in *Plant Lipid Biochemistry: Structure and Utiliziaton* (Quinn, P. J., and Harwood, J. L., eds) pp. 129-130, Portland Press, London.
12. Kinney, A. J., Hitz, W. D., and Yadav, N. S. (1990) in *Plant Lipid Biochemistry: Structure and Utilization* (Quinn, P. J., and Harwood, J. L., eds) pp. 126-128, Portland Press, London.
13. Shanklin, J., and Somerville, C. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2510-2514.
14. Thompson, G. A., Scherer, D. E., Foxall-Van Aken, S., Kenny, J. W., Young, H. L., Shintani, D. K. Kridl, J. C., and Knauf, V. C. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**. 2578-2582.
15. Nishinda, I., Beppu, T., Matsuo, T., and Murata, N. (1992) *Plant Mol. Biol.* **19**. 711-713.

16 Sato, A., Becker, C. K. and Kanuf, V. C. (1992) *Plant Physiol.* **99**, 362-363.

17. Slocombe, S. P., Cummins, I., Jarvis, R. P., and Murphy, D. J. (1992) *Plant Mol. Biol.* **20**, 151-155.

18. Taylor, M. A., Smith, S. B., Davies, H. V., and Burch, L. R. (1992) *Plant Physiol.* **100**, 533-534.

19. Cahoon, E. B., Becker, C. K. Shanklin, J., and Ohlrogge, J. B. (1994) *Plant Physiol.*, in press.

20 Singh, S., McKinney, S., Green, A. (1994) Plant Physiol. **104**, 1075.

21. Cahoon, E. B., Shanklin, J., and Ohlrogge, J. B. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11184-11188.

22. Cahoon, E. B., and Ohlrogge, J. B. (1994) *Plant Physiol.* **104**, 827-838.

23. Spencer, G. F., Kleiman, R., Miller, R. W., and Earle, F. R. (1971) *Lipids* **6**, 712-714.

24. Morrison, W. R. and Smith, L. M. (1964) *J. Lipid Res.* **5**, 600-608.

25. Cahoon, E. B., and Ohlrogge, J. B. (1994) *Plant Physiol.* **104**, 845-855.

26. Christie, W. W. (1982) *Lipid Analysis*, 2nd Ed., Pergammon Press, Oxford.

27. Rock, C. O., and Garwin, J. L. (1979) *J. Biol. Chem.* **254**, 7123-7128.

28. Hall, T. C., Ma, Y., Buchbinder, B. U., Pyne, J. W., Sun, S. M., and Bliss, F. A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3196-3200.

29. Hay, B., and Short, J. M. (1992) *Strategies* **5**, 16-18.

30. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Clonging: a Laboratory Manual.* 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

31. Francis, G. W. (1981) *Chem. Phys. Lipids* **29**, 369-374.

32. Yamamoto, K. Shibahara, A., Nakayama, T., and Kajimoto, G. (1991) *Chem. Phys. Lipdis* **60**, 39-50.

33. Gibson, K. J. (1993) *Biochim. Biophys. Acta* 1169, 231-235.

34. Lütcke, H. A., Chow, K. C., Mickel, F. S., Moss, K. A., Kern, H. F., and Scheele, G. A. (1987) *EMBO J.* **6**, 43-48.

35. Cronan Jr., J. E., and Rock, C. O. (1987) in *Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology* (Neihardt, F. C., ed) pp. 474-497. American Society of Microbiology, Washington, D.C.

36. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 60-89.

37. Schneider, G., Lindqvist, Y., Shanklin, J., and Somerville, C. (1992) *J. Mol. Biol.* **225**, 561-564.

38. Rock, C. O., and Jackowski, S. (1982) *J. Biol. Chem.* **257**, 10579-10765.

39. Knoell, H. E., and Knappe, J. (1974) *Eur. J. Biochem.* **50**, 245-252.

40. Murphy, D. J. (1992) *Trends Biotechnol.* **10**, 84-87.

41. Ohlrogge, J. B. (1992) *Plant Physiol.* **104**, 821-826.

42. Schilperoort, R. A., Hoekema, A., and Hooykaas, P. J. J. (1990) U. S. Patent No. 4,940,838.

43. Tomes, D., Bidney, D., Buising C. M. (1994) U. S. Patent No. 5,322,783.

44. Bubeck, D. M., Fehr, W. R. and Hammond, E. G. (1980) *Crop. Sci.* **29**, 652-656.

45. Perrson, C. (1985) *Cruciferae Newsletter 10*, p. 137, Scottish Crop Research Institute, Invergowerie, Dundee, Scotland.

46. Dörmann, P., Frentzen, M., and Ohlrogge, J. B. (1994) *Plant Physiol.* **104**, 839-844.

What Is Claimed Is:

1. An isolated Δ^6 - palmitoyl-acyl carrier protein desaturase.
2. A transgenic plant cell comprising heterologous DNA encoding a Δ^6 - palmitoyl-acyl carrier protein desaturase.
3. The transgenic plant cell of Claim 2 wherein said heterologous DNA is as shown in SEQ. I.D. NO.: 1.
4. The transgenic plant cell of Claim 2 wherein said Δ^6 - palmitoyl-acyl carrier protein desaturase is expressed and active.
5. The transgenic plant cell of Claim 2 as a seed or a propagate of the seed.
6. An isolated DNA encoding a Δ^6 - palmitoyl-acyl carrier protein desaturase.
7. The DNA of Claim 6 wherein said DNA encodes a *Thunbergia alata* Δ^6 - palmitoyl-acyl carrier protein desaturase.
8. The DNA of Claim 6 wherein said DNA is as shown in SEQ. ID NO.: 1
9. A mRNA derived from a DNA as shown in SEQ. I.D. NO.: 1.
10. A transgenic plant comprising heterologous DNA encoding a Δ^6 - palmitoyl-acyl carrier protein desaturase.
11. The transgenic plant of Claim 10 wherein said heterologous DNA is as shown in SEQ. I.D. NO.: 1.
12. The transgenic plant of Claim 10 wherein said Δ^6 - palmitoyl-acyl carrier protein desaturase is expressed and active.
13. A vector comprising DNA encoding a Δ^6 - palmitoyl-acyl carrier protein desaturase.
14. A method for modifying the fatty acid content of a plant seed, the method comprising transforming the plant which produces the seed with a heterologous DNA encoding a Δ^6 - palmitoyl-acyl carrier protein desaturase. and introducing a double bond at the sixth carbon atom from the carboxyl end of a 16 carbon saturated fatty acid in the plant seed through the activity of the expressed desaturase.
15. The method of Claim 14 wherein the double bond at the sixth carbon atom from the carboxyl end of the 16 carbon saturated fatty acid is

introduced by a Δ^3 - palmitoyl-acyl carrier protein desaturase from *Thunbergia alata*.

16. The method of Claim 14 further comprising oxidative cleavage of the monounsaturated fatty acid formed by the method of Claim 14.

17. A plant seed having a modified fatty acid content derived through the process of Claim 14.

18. A plant seed having a modified fatty acid content derived through the process of Claim 15.

19. A plant seed having a modified fatty acid content derived through the process of Claim 16.

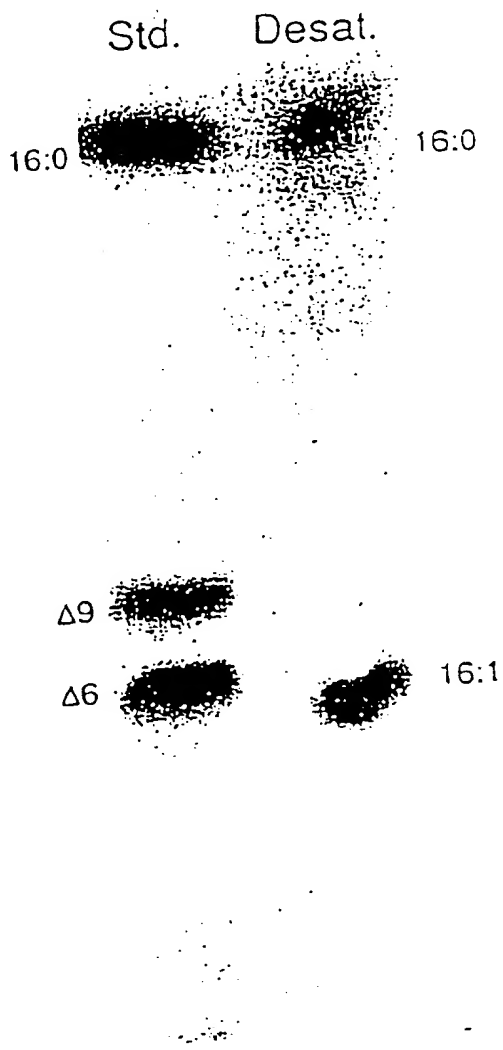


FIGURE 1

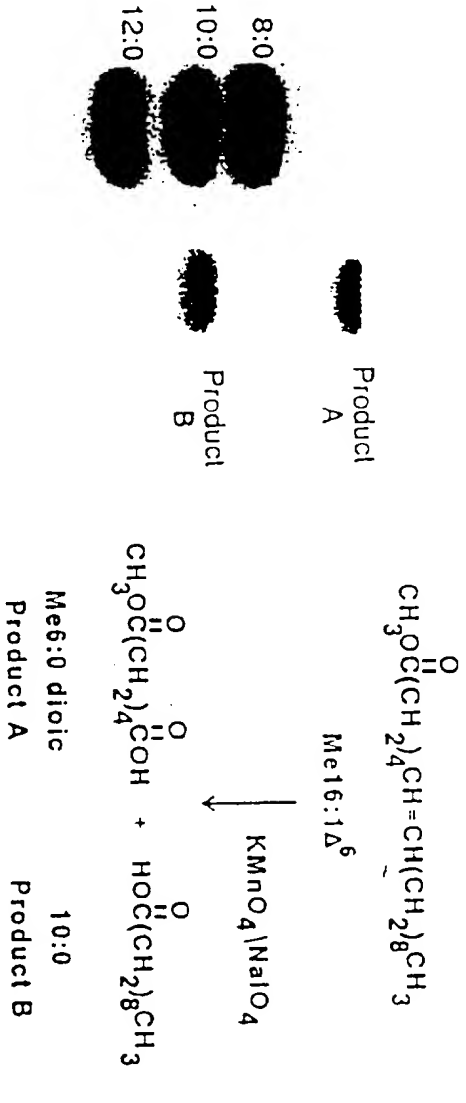

Std.
Ox.
8:0
10:0
12:0
Product A
Product B
$CH_3O\overset{O}{\overset{\|}{C}}(CH_2)_4CH=CH(CH_2)_8CH_3$
Me16:1Δ6
$KMnO_4\backslash NaIO_4$
$CH_3O\overset{O}{\overset{\|}{C}}(CH_2)_4\overset{O}{\overset{\|}{C}}OH$ + $HO\overset{O}{\overset{\|}{C}}(CH_2)_8CH_3$
Me6:0 dioic
Product A
10:0
Product B

FIGURE 2

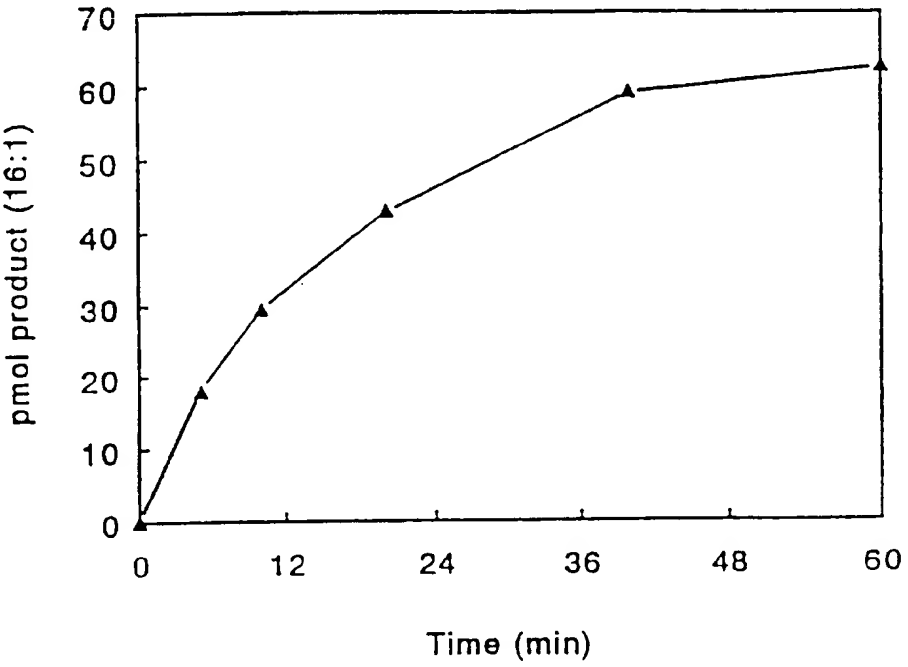


FIGURE 3

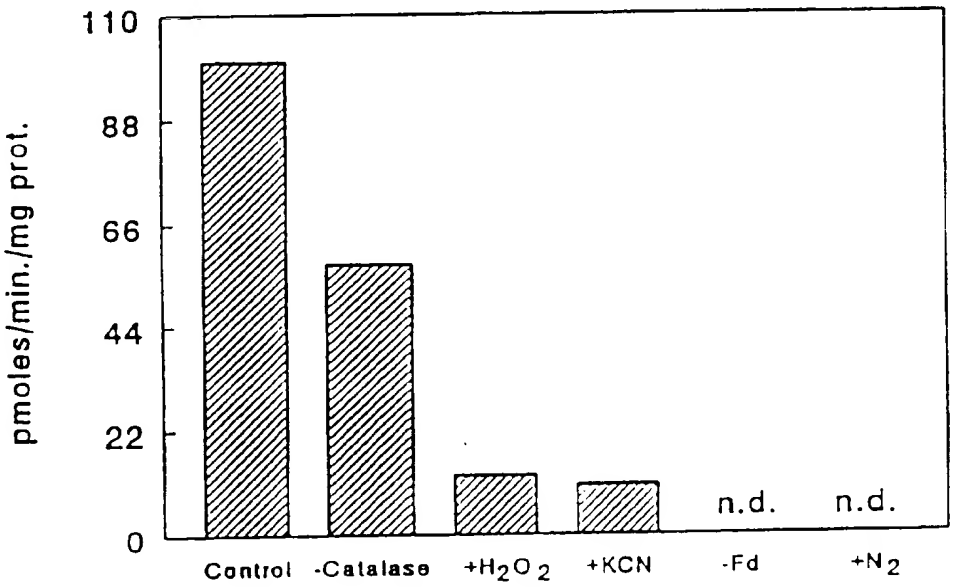

110
88
66
44
22
0
pmoles/min./mg prot.
n.d.
n.d.
Control
-Catalase
+H2O2
+KCN
-Fd
+N2

FIGURE 4

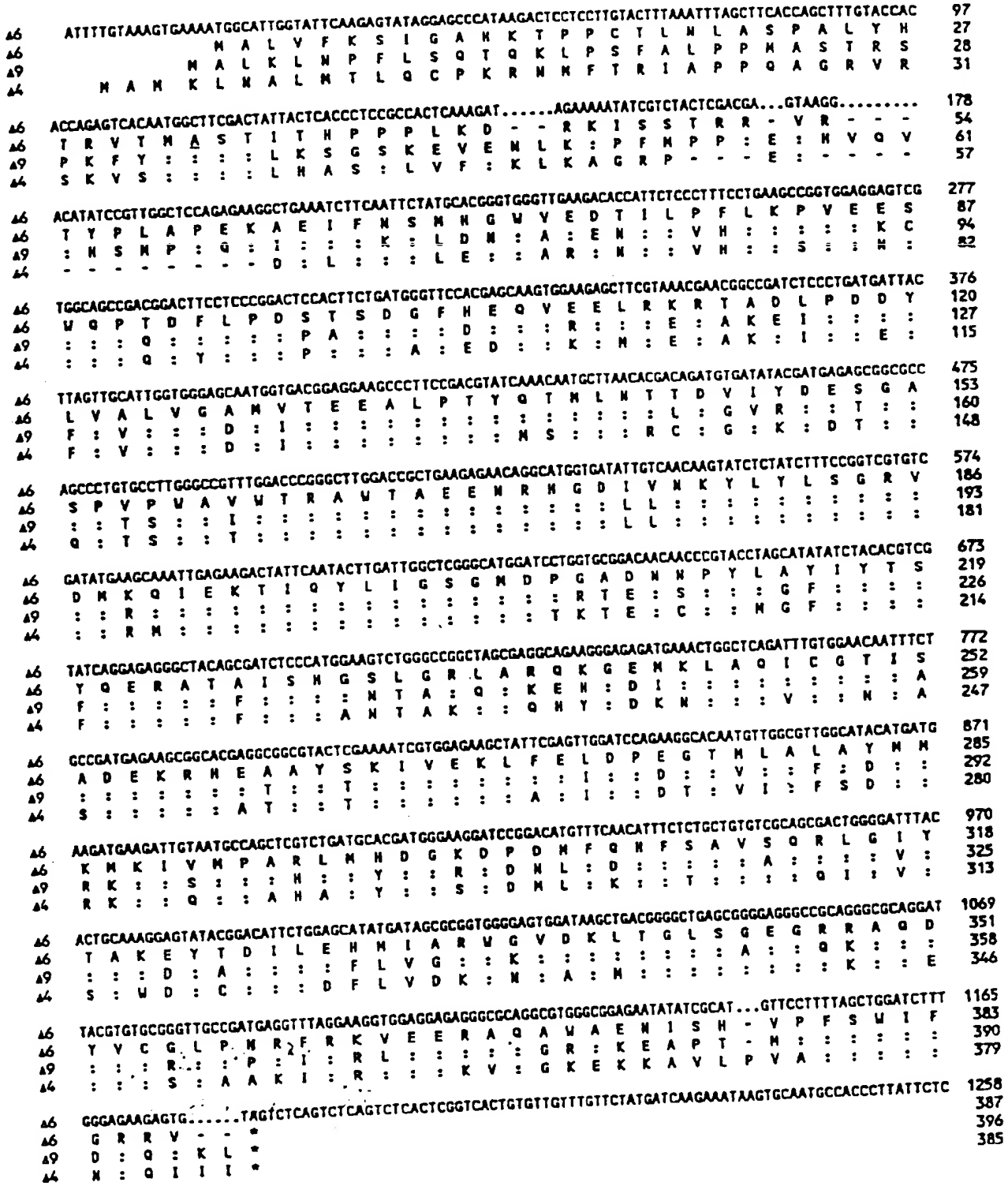


FIGURE 5

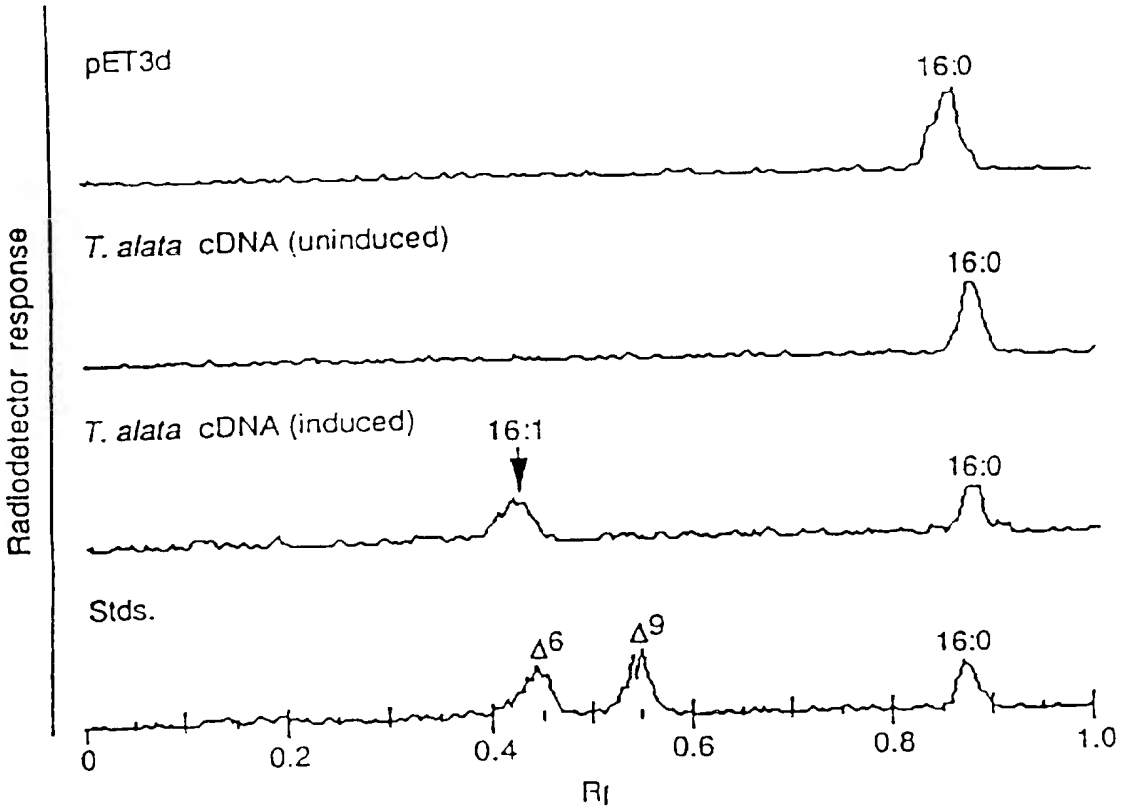

Radiodetector response
pET3d
16:0
T. alata cDNA (uninduced)
16:0
T. alata cDNA (induced)
16:1
16:0
Stds.
Δ6
Δ9
16:0
0
0.2
0.4
0.6
0.8
1.0
Rf

FIGURE 6

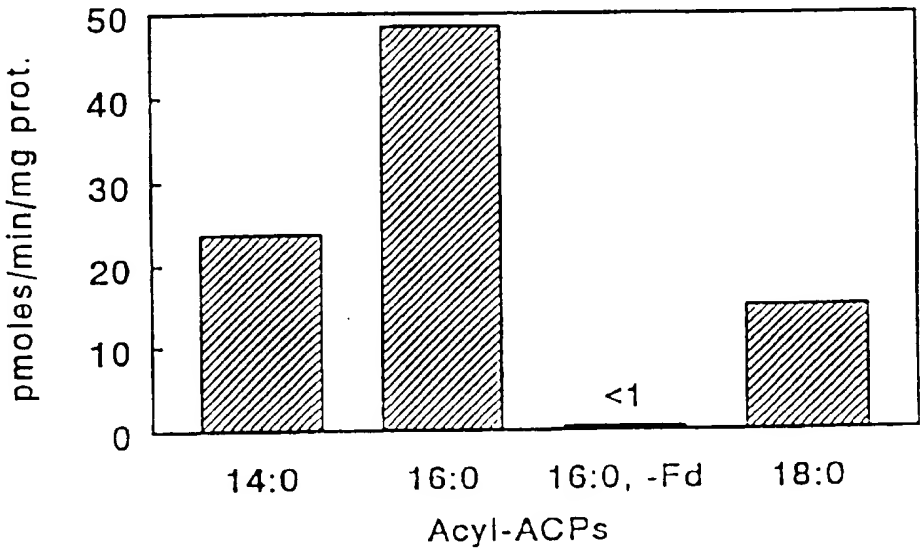


FIGURE 7

WO 96/13591 PCT/US95/13784

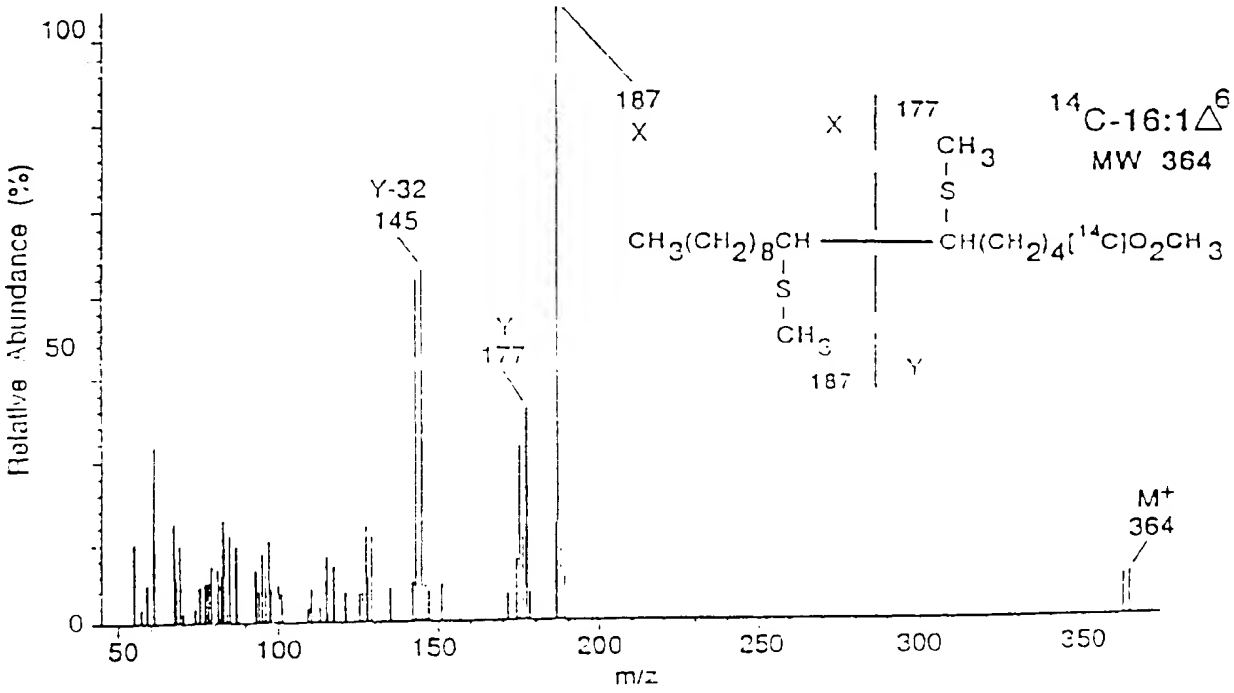


FIGURE 8

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/53, 15/82, 9/02, A01H 5/00

A3

(11) International Publication Number: WO 96/13591

(43) International Publication Date: 9 May 1996 (09.05.96)

(21) International Application Number: PCT/US95/13784

(22) International Filing Date: 25 October 1995 (25.10.95)

(30) Priority Data:
08/329,560 26 October 1994 (26.10.94) US

(71) Applicant: MICHIGAN STATE UNIVERSITY [US/US]; 238 Administration Building, East Lansing, MI 48824 (US).

(72) Inventors: CAHOON, Edgar, B.; Building 463, Brookhaven National Laboratories, Upton, NY 11973 (US). OHLROGGE, John, B.; 2123 Lagoon Drive, Okemos, MI 48864 (US).

(74) Agents: YATES, Michael, E. et al.; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).

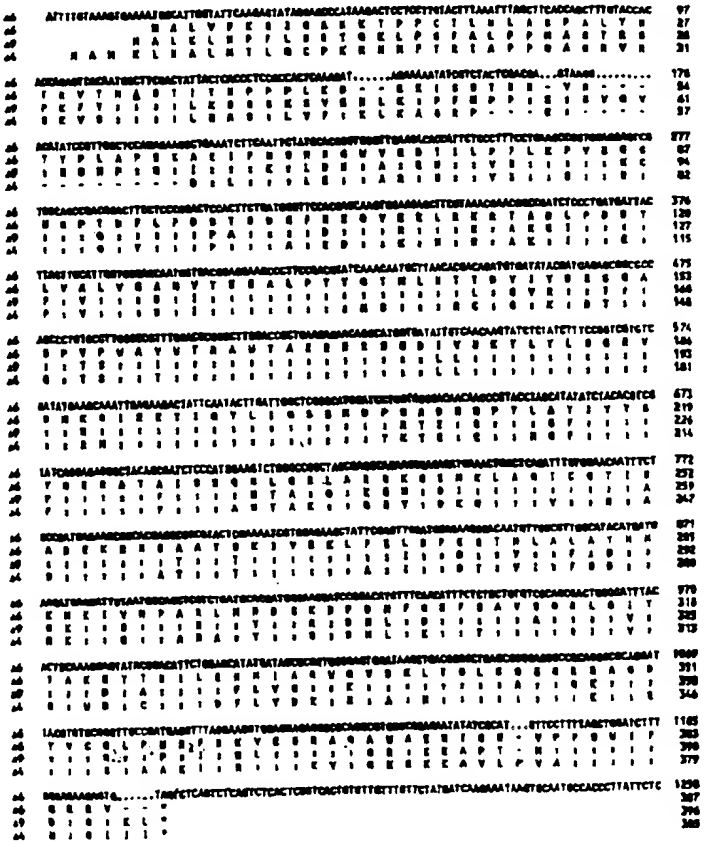
Published
With international search report.

(88) Date of publication of the international search report:
8 August 1996 (08.08.96)

(54) Title: METHODS AND COMPOSITIONS RELATING TO PLANT Δ^6 PALMITOYL-ACYL CARRIER PROTEIN DESATURASE

(57) Abstract

A plant Δ^6 palmitoyl-acyl carrier protein desaturase, the gene encoding the desaturase, and transgenic plants and plant cells containing the heterologous DNA encoding the desaturase are described. The desaturase introduces a double bond at the sixth carbon atom from the carboxyl end of a 16 carbon saturated fatty acid, and is therefore useful in production of plant seeds having a modified fatty acid profile.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SI	Slovenia
SK	Slovakia
SN	Senegal
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

INTERNATIONAL SEARCH REPORT

PCT/US 95/13784

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N15/82 C12N9/02 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 122, no. 13, 27 March 1995 Columbus, Ohio, US; abstract no. 156368, CAHOON, EDGAR BENJAMIN: "Synthesis and metabolism of .DELTA.6 monosaturated fatty acids in developing seed of Umbelliferae species and Thunbergia alata" XP002000650 see abstract & DISSERTATION (1994) 219 PP. AVAIL.: UNIV. MICROFILMS INT., ORDER NO. DA9431218 FROM: DISS. ABSTR. INT. B 1995, 55(7), 2482 , --- -/--	1,6-9,13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
16 April 1996	09.05.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	J. BIOL. CHEM. (1994), 269(44), 27519-26, 4 November 1994, XP002000645 CAHOON, EDGAR B., ET AL.: ".DELTA.6 hexadecenoic acid is synthesized by the activity of a soluble .DELTA.6 palmitoyl-acyl carrier protein desaturase in Thunbergia alata endosperm" see the whole document	1,6-9,13
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, 1992, WASHINGTON US, pages 11184-11188, XP002000646 CAHOON, E.B., ET AL.: "Expression of a coriander desaturase results in petroselenic acid production in transgenic tobacco" see the whole document	1-19
A	PLANT PHYSIOLOGY, vol. 106, October 1994, pages 807-808, XP002000647 CAHOON, E.B., ET AL.: "cDNAs for isoforms of the delta9-steaoryl-acyl carrier protein desaturase from Thunbergia alata endosperm" see the whole document	1-19
A	PLANT MOLECULAR BIOLOGY, vol. 26, October 1994, pages 631-642, XP002000648 SCHMIDT, H., ET AL.: "Purification and PCR-based cloning of a plastidial n-6 desaturase" see the whole document	1-19
A	LIPIDS, vol. 6, 1971, pages 712-714, XP002000649 SPENCER, G.F., ET AL.: "Occurrence of cis-6-hexadecenoic acid as the major component of Thunbergia alata seed oil" see the whole document	1-19
A	WO,A,93 11245 (DU PONT) 10 June 1993 see page 85 - page 88	1-19
A	WO,A,94 01565 (MINI AGRICULTURE & FISHERIES) 20 January 1994 see the whole document	1-19

2

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9311245	10-06-93	AU-B-	3228893	28-06-93
		CA-A-	2124673	10-06-93
		EP-A-	0616644	28-09-94
		JP-T-	7501701	23-02-95
WO-A-9401565	20-01-94	AU-B-	4508393	31-01-94
		EP-A-	0603371	29-06-94

United States Patent *6,020,122*
Okasinski , et al. **February 1, 2000**

Hepatitis C virus second envelope (HCV-E2) glycoprotein expression system

Abstract

This invention provides a novel mammalian expression system that is capable of generating high levels of expressed hepatitis C virus (HCV) proteins which have previously proved difficult to express due to their non-secretory properties. In particular, the invention provides a plasmid for the expression of the HCV second envelope protein (E2) designated p577. This plasmid encodes a recombinant protein comprising the immunoglobulin signal peptide and amino acids 388-664 of the HCV E2 glycoprotein. This unique expression system produces high levels of HCV proteins that are properly processed, glycosylated, and folded.

Inventors: **Okasinski; Gregory F.** (Wadsworth, IL); **Schaefer; Verlyn G.**(Libertyville, IL); **Suhar; Thomas S.** (Lindenhurst, IL); **Lesniewski; Richard R.** (Kenosha, WI)
Assignee: **Abbott Laboratories** (Abbott Park, IL)
Appl. No.: **478073**
Filed: **June 7, 1995**

Current U.S. Class: **435/5**; 424/189.1; 424/228.1; 435/69.1; 435/71.1; 530/350
Intern'l Class: C12Q 001/70
Field of Search: 435/4,5,7.1,69.1,320.1 424/189.1,192.1,228.1,201.1 536/23.72

References Cited [Referenced By]

U.S. Patent Documents

5322769 Jun., 1994 Bolling et al..

Other References

Newman et al., 1995, Vaccine Design: The Subunit and Adjuvant Approach, Powell et al., eds., Plenum Press, New York, pp. 1-42.
Graham et al., 1995, New Engl. J. Med. 333:1331-1339.
Prince, 1994, FEMS Microbiol. Rev. 14:273-278.
Lesniewski et al., 1995, J. Med. Virol. 45:415-422.
Yokosuka et al., 1993, Gut supplement:S64-S65.
Harada et al., 1995, J. Gen. Virol. 76:1223-1231.

Primary Examiner: Scheiner; Laurie
Assistant Examiner: Parkin; Jeffrey S.
Attorney, Agent or Firm: Becker; Cheryl L., Porembski; Priscilla

Parent Case Text

RELATED APPLICATIONS

This application is related to pending U.S. patent application Ser. No. 08/188,281, filed Jan. 28, 1994, entitled "Mammalian Expression Systems for Hepatitis C Virus Envelope Genes" and to pending U.S. patent application Ser. No. 08/144,099, filed Oct. 28, 1993, entitled "Mammalian Expression Systems for Hepatitis C Virus", which is a continuation of U.S. Ser. No. 07/830,024, both of which enjoy common ownership and are incorporated herein by reference.

Claims

What is claimed is:

1. A method for detecting the presence of an anti-hepatitis C virus (HCV) antibody in a test sample comprising the following steps:

(a) contacting at least one antigen specific for said antibody with a test sample under conditions that facilitate antigen/antibody complex formation, wherein said antigen is the recombinant glycosylated HCV protein encoded by plasmid 577 (p577) having the structure shown in FIG. 1 and has been isolated and purified from mammalian host cells transfected with p577; and

(b) contacting the complex of step (a) with an indicator reagent comprising said antigen conjugated to an signal generating compound under conditions that facilitate antigen/antibody/indicator reagent complex formation,

wherein the signal generated is an indication of the presence of said anti-HCV antibody in said test sample.

2. A test kit for the detection of hepatitis C virus (HCV)-specific antibody in a test sample, wherein said kit comprises at least one container containing an isolated and purified HCV antigen, said antigen consisting of the recombinant, glycosylated HCV protein encoded by the plasmid designated p577 having the structure shown in FIG. 1.

3. A composition comprising a recombinant, glycosylated hepatitis C virus (HCV) protein, wherein said protein is encoded by the plasmid designated p577 having the structure shown in FIG. 1 and said protein has been isolated and purified from mammalian host cells transfected with p577.

4. An isolated and purified plasmid encoding a recombinant, glycosylated hepatitis C virus (HCV) antigen, wherein said plasmid has the designation p577 and the structure set forth in FIG. 1.

Description

BACKGROUND OF THE INVENTION

This invention relates generally to a mammalian expression system, and more particularly, relates to a mammalian expression system capable of generating recombinant proteins not heretofore generated at such high levels due to the non-secretor nature of the gene. The recombinant proteins are expressed in culture medium as well as in mammalian cells.

The introduction of the first-generation hepatitis C virus (HCV) enzyme immunoassays (EIAs) (HCV 1.0 EIAs) as screening assays in 1989 and second-generation HCV EIAs in 1992 (HCV 2.0 EIAs) has dramatically reduced the incidence of post-transfusion HCV (PT-HCV) infection in those countries where routine screening of donated blood products is performed. Antibodies to HCV are detected using recombinant proteins derived from the core, NS3 (viral protease) and NS4 (function unknown) genes of the virus. HCV third-generation EIAs (HCV 3.0 EIAs) which include an additional antigen from the NS5 region (containing the viral polymerase and a second unknown function) now are available and in use in several countries. HCV envelope antigens have not been used in these assays.

Difficulties in the expression and purification of the putative HCV viral envelope proteins (E1, E2) have prevented detailed research and possible incorporation of these proteins as targets in blood screening assays. There may be several reasons for the difficulties encountered in getting a cell to synthesize a heterologous protein and subsequently, to detect and recover the protein. For example, the heterologous gene may not be efficiently transcribed into messenger RNA (mRNA). Also, the mRNA may be unstable and degrade prior to translation into the protein. In addition, the ribosome binding site (RBS) present on the mRNA may only poorly initiate translation. The heterologous protein produced may be unstable in the cell or it may be toxic to the cell. If no antibodies to the protein are available or if there is no other way to assay for the protein, it may be difficult to detect the synthesized protein. Lastly, even if the protein is produced, it may be difficult to purify.

Fusion systems provide a means of solving many of the aforementioned problems. The "carrier" portion of the hybrid gene, typically found on the 5' end of the gene, provides the regulatory regions for transcription and translation as well as providing the genetic code for a peptide which facilitates detection (Shuman et al., J. Biol. Chem. 255:168 [1980]) and/or purification (Moks et al., Bio/Technology 5:379 [1987]). Frequently, potential proteolytic cleavage sites are engineered into the fusion protein to allow for the removal of the homologous peptide portion (de Geus et al., Nucleic Acids Res. 15:3743 [1987]; Nambiar et al., Eur. J. Biochem. 163:67 [1987]; Imai et al., J. Biochem. 100:425 [1986]).

When selecting a carrier gene for a fusion system, in addition to detectability and ease of purification, it would be extremely advantageous to start with a highly expressed gene. Expression is the result of not only efficient transcription and translation but also protein stability and benignity (the protein must not harm or inhibit the cell host). Such expression is advantageous because it can enable the production of such fusion proteins for use in assays. In genes where such expression is not possible, it would be advantageous to provide a system whereby a non-secretor gene can secrete, or express, protein in sufficient amounts to be useful in commercial assays or for other purposes such as for vaccine production.

SUMMARY OF THE INVENTION

This invention provides a novel mammalian expression system that is capable of generating high levels of expressed proteins which proteins heretofore have been difficult to express due to the non-secretor nature of the gene. In particular, the invention provides a plasmid for the expression of the HCV E2 antigen. This unique expression systems allow for the production of high levels of HCV proteins, allowing to the proper processing, glycosylation and conformation (folding) of the viral protein(s) in the system. In particular, the present invention provides the plasmid 577. HCV E2 fusion protein, expressed

from plasmid 577 in the mammalian expression system of the invention, can be recovered extracelluarly as well as intracellularly.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graphic representation of plasmid 577.

FIG. 2 is the DNA sequence of HCV E2 antigen expression cassette.

FIG. 3 (labelled translation figure) shows a conceptual translation of the HCV E2 gene and the signal protease cleavage site wherein " - - - " denotes a signal peptidease cleavage site.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides ways to produce glycosylated HCV E2 fusion proteins expressed in mammalian expression systems. These glycosylated proteins have utility for a variety of applications, including, for example, assay systems for screening and prognostic applications, and as vaccine preparations. These HCV viral envelope proteins expressed in mammalian cells also allow for inhibitor studies including elucidation of specific viral attachment sites or sequences and/or viral receptors on susceptible cell types, for example, liver cells and the like.

The procurement of specific expression clones developed as described herein in mammalian expression systems provides antigens for diagnostic assays which can aid in determining the stage of HCV infection, such as, for example, acute versus on-going or persistent infections, and/or recent infection versus past exposure. These specific expression clones also provide prognostic markers for resolution of disease such as to distinguish resolution of disease from chronic hepatitis caused by HCV. It is contemplated that earlier seroconversion to glycosylated structural antigens may be detectable by using proteins produced in these mammalian expression systems. Antibodies, both monoclonal and polyclonal, also may be produced from the proteins derived from these mammalian expression systems which then in turn may be used for diagnostic, prognostic and therapeutic applications.

Proteins produced from these mammalian expression systems, as well as reagents produced from these proteins, can be provided in the form of a kit with one or more containers such as vials or bottles, with each container containing a separate reagent such as a monoclonal antibody, or a cocktail of monoclonal antibodies, or a recombinant protein, packaged as test kits for convenience in performing assays. Other aspects of the present invention include a recombinant protein comprising an HCV epitope attached to a solid phase and an antibody to an HCV epitope attached to a solid phase. Also included are methods for producing a recombinant protein containing an HCV epitope by incubating host cells transformed with a mammalian expression vector containing a sequence encoding a polypeptide containing an HCV epitope under conditions which allow expression of the polypeptide, and a polypeptide containing an HCV epitope produced by this method.

The present invention provides assays which utilize the recombinant proteins provided by the invention, as well as the antibodies described herein in various formats, any of which may employ a signal generating compound which generates a measurable signal in the assay. Assays which do not utilize signal generating compounds to provide a means of detection also are provided. All of the assays described generally detect either antigen or antibody, or both, and include mixing a test sample with at least one reagent provided herein to form at least one antigen/antibody complex and detecting the presence of the complex. These assays are described in detail herein.

Vaccines for treatment of HCV infection comprising an immunogenic peptide obtained from a mammalian expression system containing envelope genes from HCV as described herein are included in the present invention. Also included in the present invention is a method for producing antibodies to HCV comprising administering to an individual an isolated immunogenic polypeptide containing an HCV epitope in an amount sufficient to produce an immune response in the inoculated individual.

The term "test sample" refers to a component of an individual's body which is the source of the antibodies of interest. These components are well known in the art and include biological samples which

can be tested by the methods described herein. Examples of test samples include human and animal body fluids such as whole blood, serum, plasma, cerebrospinal fluid, urine, lymph fluids, and various external sections of the respiratory, intestinal and genitourinary tracts, tears, saliva, milk, white blood cells, myelomas and the like, biological fluids such as cell culture supernatants, fixed tissue specimens and fixed cell specimens.

After preparing the recombinant proteins as described by the present invention, these recombinant proteins can be used to develop unique assays as described herein to detect either the presence of antigen or antibody to HCV. These compositions also can be used to develop monoclonal and/or polyclonal antibodies with a specific recombinant protein which specifically binds to the immunological epitope of HCV. Also, it is contemplated that recombinant proteins made by the method described herein can be used to develop vaccines by following methods known in the art.

Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in or suspension in liquid prior to injection also may be prepared. The preparation may be emulsified. or the protein may be encapsulated in liposomes. The active immunogenic ingredients often are mixed with pharmacologically acceptable excipients which are compatible with the active ingredient. Suitable excipients include but are not limited to water, saline, dextrose, glycerol, ethanol and the like; combinations of these excipients in various amounts also may be used. The vaccine also may contain small amounts of auxiliary substances such as wetting or emulsifying reagents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. For example, such adjuvants can include aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-DMP), N-acetyl-nornuramyl-L-alanyl-D-isoglutamine (CGP 11687, also referred to as nor-MPD), N-acetylmuramyul-L-alanyl-D-isoglutaminyl-L-alanine-2-(1`2`-dipalmitoyl-sn -glycero-3-hydroxphosphoryloxy)-ethylamine (CGP19835A, also referred to as MTP-PE), and RIBI (MPL+TDM+CWS) in a 2% squalene/Tween-80.RTM. emulsion. The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic polypeptide containing an HCV antigenic sequence resulting from administration of this polypeptide in vaccines which also are comprised of the various adjuvants.

The vaccines usually are administered by intraveneous or intramuscular injection. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include but are not limited to polyalkylene glycols or triglycerides. Such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10%, preferably, about 1% to about 2%. Oral formulation include such normally employed excipients as, for example pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions may take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 10% to about 95% of active ingredient, preferably about 25% to about 70%.

The proteins used in the vaccine may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts such as acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric, maleic, and others known to those skilled in the art. Salts formed with the free carboxyl groups also may be derived from inorganic bases such as sodium, potassium, ammonium, calcium or ferric hydroxides and the like, and such organic bases such as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine procaine, and others known to those skilled in the art.

Vaccines are administered in a way compatible with the dosage formulation, and in such amounts as will be prophylactically and/or therapeutically effective. The quantity to be administered generally is in the range of about 5 micrograms to about 250 micrograms of antigen per dose, and depends upon the subject to be dosed, the capacity of the subject's immune system to synthesize antibodies, and the degree of protection sought. Precise amounts of active ingredient required to be administered also may depend upon the judgment of the practitioner and may be unique to each subject. The vaccine may be given in a single or multiple dose schedule. A multiple dose is one in which a primary course of vaccination may

be with one to ten separate doses, followed by other doses given at subsequent time intervals required to maintain and/or to reenforce the immune response, for example, at one to four months for a second dose, and if required by the individual, a subsequent dose(s) after several months. The dosage regimen also will be determined, at least in part, by the need of the individual, and be dependent upon the practitioner's judgment. It is contemplated that the vaccine containing the immunogenic HCV envelope antigen(s) may be administered in conjunction with other immunoregulatory agents, for example, with immune globulins.

The expression of a gene coding for a protein of interest using a DNA cloning vehicle which includes (a) expression control regions, (b) a region coding for the rabbit immunoglobulin heavy chain gamma secretion signal sequence, (c) bacterial enzyme for selection in eukaryotic cells, (d) an amplification system suitable for enhanced expresision in eukaryotic cells, and (e) a region coding for the protein of interest generally is described herein. The cloning vehicles described herein are capable of expressing fusion proteins; that is, immunoglobulin signal peptides sequences and adjacent immunoglobulin coding sequences fused to heterologous protein at commercially useful levels. FIG. 1 shows generically the features of a plasmid useful for production of fusion proteins used in the methods of this invention. The plasmid in FIG. 1 is disclosed as a series of assembled fragments with sections 1 to 13. The accession numbers of the sections refer to Genbank.RTM. accession numbers. The plasmid includes a control region (described hereinbelow), followed by a gene encoding an immunoglobulin signal peptide and adjacent immunoglobilin coding sequences which are linked to a gene coding for a heterologous protein of interest. Please note that slight sequence variations may occur and may have occurred when constructing the plasmid.

TABLE 1

Plasmid Figure Legend Construction Plasmid 577, 10,186 base pairs double stranded DNA SECTION	DESCRIPTION
1	(NT 4361-2067 OF PBR322 ACCESSION J02224)
2	(NT 2249-1624 HSV-1 ACCESSION J02224 NT)
3	(NT 2518-1519 Tn5 ACCESSION NOS U00004 L19385)
4	(NT 460-210 HSV-1 ACCESSION J02224)
5	(NT 272-1, 5243-5173 SV40)
6	(NT 1-701 MOUSE DHFR ACCESSION L26316)
7	(NT 4714-4100 SV40 ACCESSION V08380)
8	(NT 272-1, 5243-5173 5V40)
9	(NT 1-77 DNA Sequence Figure SYNTHETIC DNA RABBITT IgG HEAVY CHAIN LEADER (HCL))
10	(NT 78-938 DNA Sequence Figure HCV E2 antigen PCR product)
11	(HBV ENHANCER NT 2373-2811 ACCESSION NO. X02763 WITH G AT NT. 2976 AND T AT NT. 2654)
12	(NT 3688-5468 HSV1 ACCESSION NO. NT 3687-5468)
13	(NT 2536-1785 SV40 ACCESSION V08380)

Insertion of heterologous genes into a plasmid as described in FIG. 1 can be accomplished with various techniques known to those in the art. These fusion proteins can be utilized in various assay formats as capture reagents or protein binders in numerous ways. After preparing the recombinant proteins as described herein, the recombinant proteins can be used to develop unique assays as described herein to detect either the presence of a specific binding member of a specific binding pair. These recombinant proteins also can be used to develop monoclonal and/or polyclonal antibodies with a specific recombinant protein or synthetic peptide which specifically binds to the specific binding member of a specific binding pair. The fusion proteins described herein also can be used as the active ingredient of a vaccine.

Vaccine Preparation

Vaccines may be prepared from one or more immunogenic polypeptides derived from nucleic acid sequences of interest or from the genome of interest to which they correspond. Vaccines may comprise recombinant polypeptides containing epitope(s) of interest. These polypeptides may be expressed in bacteria, yeast or mammalian cells, or alternatively may be isolated from viral preparations. It also is anticipated that various structural proteins may contain epitopes of interest which give rise to protective anti-epitope antibodies. Synthetic peptides therefore also can be utilized when preparing these vaccines. Thus, polypeptides containing at least one epitope of interest may be used, either singly or in combinations, in these vaccines. It also is contemplated that nonstructural proteins as well as structural proteins may provide protection against viral pathogenicity, even if they do not cause the production of neutralizing antibodies.

Considering the above, multivalent vaccines may comprise one or more structural proteins, and/or one or more nonstructural proteins. These vaccines may be comprised of, for example, recombinant polypeptides expressed by the plasmid of the invention and/or polypeptides isolated from the virions and/or synthetic peptides. These immunogenic epitopes can be used in combinations, i.e., as a mixture of recombinant proteins, synthetic peptides and/or polypeptides isolated from the virion; these may be administered at the same or different time. Additionally, it may be possible to use inactivated viruses in vaccines. Such inactivation may be be preparation of viral lysates, or by other means known in the art to cause inactivation of hepatitis-like viruses, for example, treatment with organic solvents or detergents, or treatment with formalin. Attenuated viral strain preparation also is disclosed in the present invention. It is contemplated that some of the proteins may cross-react with other known viruses, and thus that shared epitopes may exist between the virus of interest and other viruses which would then give rise to protective antibodies against one or more of the disorders caused by these pathogenic agents. It is contemplated that it may be possible to design multiple purpose vaccines based upon this belief.

The preparation of vaccines which contain at least one immunogenic peptide as an active ingredient is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in or suspension in liquid prior to injection also may be prepared. The preparation may be emulsified or the protein may be encapsulated in liposomes. The active immunogenic ingredients often are mixed with pharmacologically acceptable excipients which are compatible with the active ingredient. Suitable excipients include but are not limited to water, saline, dextrose, glycerol, ethanol and the like; combinations of these excipients in various amounts also may be used. The vaccine also may contain small amounts of auxiliary substances such as wetting or emulsifying reagents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. For example, such adjuvants can include aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-DMP), N-acetyl-nornuramyl-L-alanyl-D-isoglutamine (CGP 11687, also referred to as nor-MDP), N-acetylmuramyul-L-alanyl-D-isoglutaminyl-L-alanine-2-(1`2`-dipalmitoyl-sn -glycero-3-hydroxphosphoryloxy)-ethylamine (CGP 19835A, also referred to as MTP-PE), and REBI (MPL+TDM+CWS) in a 2% squalene/Tween-80.RTM. emulsion. The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic polypeptide containing an antigenic sequence produced by the plasmid disclosed herein, resulting from administration of this polypeptide in vaccines which also are comprised of the various adjuvants.

The vaccines usually are administered by intraveneous or intramuscular injection. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include but are not limited to polyalkylene glycols or triglycerides. Such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10%, preferably, about 1% to about 2%. Oral formulation include such normally employed excipients as, for example pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions may take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 10% to about 95% of active ingredient, preferably about 25% to about 70%.

The proteins used in the vaccine may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts such as acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as hydrochloric or phosphoric acids, or such

organic acids such as acetic, oxalic, tartaric, maleic, and others known to those skilled in the art. Salts formed with the free carboxyl groups also may be derived from inorganic bases such as sodium, potassium, ammonium, calcium or ferric hydroxides and the like, and such organic bases such as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine procaine, and others known to those skilled in the art.

Vaccines are administered in a way compatible with the dosage formulation, and in such amounts as will be prophylactically and/or therapeutically effective. The quantity to be administered generally is in the range of about 5 micrograms to about 250 micrograms of antigen per dose, and depends upon the subject to be dosed, the capacity of the subject's immune system to synthesize antibodies, and the degree of protection sought. Precise amounts of active ingredient required to be administered also may depend upon the judgment of the practitioner and may be unique to each subject. The vaccine may be given in a single or multiple dose schedule. A multiple dose is one in which a primary course of vaccination may be with one to ten separate doses, followed by other doses given at subsequent time intervals required to maintain and/or to reinforce the immune response, for example, at one to four months for a second dose, and if required by the individual, a subsequent dose(s) after several months. The dosage regimen also will be determined, at least in part, by the need of the individual, and be dependent upon the practitioner's judgment. It is contemplated that the vaccine containing the immunogenic antigen(s) prepared as described herein may be administered in conjunction with other immunoregulatory agents, for example, with immune globulins.

Assay Formats

It is contemplated that the reagent employed for the assay can be provided in the form of a kit with one or more containers such as vials or bottles, with each container containing a separate reagent such as a monoclonal antibody, or a cocktail of monoclonal antibodies, or a recombinant protein employed in the assay.

"Analyte," as used herein, is the substance to be detected which may be present in the test sample. The analyte can be any substance for which there exists a naturally occurring specific binding member (such as, an antibody), or for which a specific binding member can be prepared. Thus, an analyte is a substance that can bind to one or more specific binding members in an assay. "Analyte" also includes any antigenic substances, haptens, antibodies, and combinations thereof. As a member of a specific binding pair, the analyte can be detected by means of naturally occurring specific binding partners (pairs) such as the use of intrinsic factor protein as a member of a specific binding pair for the determination of Vitamin B12, the use of folate-binding protein to determine folic acid, or the use of a lectin as a member of a specific binding pair for the determination of a carbohydrate. The analyte can include a protein, a peptide, an amino acid, a hormone, a steroid, a vitamin, a drug including those administered for therapeutic purposes as well as those administered for illicit purposes, a bacterium, a virus, and metabolites of or antibodies to any of the above substances. The details for the preparation of such antibodies and the suitability for use as specific binding members are well known to those skilled in the art. Viruses which can be tested include hepatitis-causing viruses (for example, hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis delta, and hepatitis E virus, and hepatitis GB viruses), human immunodeficiency viruses (such as HIV-1, HIV-2), the HTLV-I and HTLV-II viruses, and the like.

The present invention provides assays which utilize specific binding members. A "specific binding member," as used herein, is a member of a specific binding pair. That is, two different molecules where one of the molecules through chemical or physical means specifically binds to the second molecule. Therefore, in addition to antigen and antibody specific binding pairs of common immunoassays, other specific binding pairs can include biotin and avidin, carbohydrates and lectins, complementary nucleotide sequences, effector and receptor molecules, cofactors and enzymes, enzyme inhibitors and enzymes, and the like. Furthermore, specific binding pairs can include members that are analogs of the original specific binding members, for example, an analyte-analog. Immunoreactive specific binding members include antigens, antigen fragments, antibodies and antibody fragments, both monoclonal and polyclonal, and complexes thereof, including those formed by recombinant DNA molecules. The term "hapten", as used herein, refers to a partial antigen or non-protein binding member which is capable of binding to an antibody, but which is not capable of eliciting antibody formation unless coupled to a carrier protein.

A "capture reagent", as used herein, refers to an unlabeled specific binding member which is specific either for the analyte as in a sandwich assay, for the indicator reagent or analyte as in a competitive assay, or for an ancillary specific binding member, which itself is specific for the analyte, as in an indirect assay. The capture reagent can be directly or indirectly bound to a solid phase material before the performance of the assay or during the performance of the assay, thereby enabling the separation of immobilized complexes from the test sample.

"Indicator Reagents" may be used in the various assay formats described herein. The "indicator reagent" comprises a "signal generating compound" (label) which is capable of generating a measurable signal detectable by external means conjugated (attached) to a specific binding member for the analyte. "Specific binding member" as used herein means a member of a specific binding pair. That is, two different molecules where one of the molecules through chemical or physical means specifically binds to the second molecule. In addition to being an antibody member of a specific binding pair for the analyte, the indicator reagent also can be a member of any specific binding pair, including either hapten-anti-hapten systems such as biotin or anti-biotin, avidin or biotin, a carbohydrate or a lectin, a complementary nucleotide sequence, an effector or a receptor molecule, an enzyme cofactor and an enzyme, an enzyme inhibitor or an enzyme, and the like. An immunoreactive specific binding member can be an antibody, an antigen, or an antibody/antigen complex that is capable of binding either to the analyte as in a sandwich assay, to the capture reagent as in a competitive assay, or to the ancillary specific binding member as in an indirect assay.

The various "signal generating compounds" (labels) contemplated include chromogens, catalysts such as enzymes, luminescent compounds such as fluorescein and rhodamine, chemiluminescent compounds, radioactive elements, and direct visual labels. Examples of enzymes include alkaline phosphatase, horseradish peroxidase, beta-galactosidase, and the like. The selection of a particular label is not critical, but it will be capable of producing a signal either by itself or in conjunction with one or more additional substances.

"Solid phases" ("solid supports") are known to those in the art and include the walls of wells of a reaction tray, test tubes, polystyrene beads, magnetic beads, nitrocellulose strips, membranes, microparticles such as latex particles, and others. The "solid phase" is not critical and can be selected by one skilled in the art. Thus, latex particles, microparticles, magnetic or non-magnetic beads, membranes, plastic tubes, walls of microtiter wells, glass or silicon chips and sheep red blood cells are all suitable examples. Suitable methods for immobilizing peptides on solid phases include ionic, hydrophobic, covalent interactions and the like. A "solid phase", as used herein, refers to any material which is insoluble, or can be made insoluble by a subsequent reaction. The solid phase can be chosen for its intrinsic ability to attract and immobilize the capture reagent. Alternatively, the solid phase can retain an additional receptor which has the ability to attract and immobilize the capture reagent. The additional receptor can include a charged substance that is oppositely charged with respect to the capture reagent itself or to a charged substance conjugated to the capture reagent. As yet another alternative, the receptor molecule can be any specific binding member which is immobilized upon (attached to) the solid phase and which has the ability to immobilize the capture reagent through a specific binding reaction. The receptor molecule enables the indirect binding of the capture reagent to a solid phase material before the performance of the assay or during the performance of the assay. The solid phase thus can be a plastic, derivatized plastic, magnetic or non-magnetic metal, glass or silicon surface of a test tube, microtiter well, sheet, bead, microparticle, chip, and other configurations known to those of ordinary skill in the art.

It is contemplated and within the scope of the invention that the solid phase also can comprise any suitable porous material with sufficient porosity to allow access by detection antibodies and a suitable surface affinity to bind antigens. Microporous structures are generally preferred, but materials with gel structure in the hydrated state may be used as well. Such useful solid supports include:

natural polymeric carbohydrates and their synthetically modified, cross-linked or substituted derivatives, such as agar, agarose, cross-linked alginic acid, substituted and cross-linked guar gums, cellulose esters, especially with nitric acid and carboxylic acids, mixed cellulose esters, and cellulose ethers; natural polymers containing nitrogen, such as proteins and derivatives, including cross-linked or modified

gelatins; natural hydrocarbon polymers, such as latex and rubber; synthetic polymers which may be prepared with suitably porous structures, such as vinyl polymers, including polyethylene, polypropylene, polystyrene, polyvinylchloride, polyvinylacetate and its partially hydrolyzed derivatives, polyacrylamides, polymethacrylates, copolymers and terpolymers of the above polycondensates, such as polyesters, polyamides, and other polymers, such as polyurethanes or polyepoxides; porous inorganic materials such as sulfates or carbonates of alkaline earth metals and magnesium, including barium sulfate, calcium sulfate, calcium carbonate, silicates of alkali and alkaline earth metals, aluminum and magnesium; and aluminum or silicon oxides or hydrates, such as clays, alumina, talc, kaolin, zeolite, silica gel, or glass (these materials may be used as filters with the above polymeric materials); and mixtures or copolymers of the above classes, such as graft copolymers obtained by initializing polymerization of synthetic polymers on a pre-existing natural polymer. All of these materials may be used in suitable shapes, such as films, sheets, or plates, or they may be coated onto or bonded or laminated to appropriate inert carriers, such as paper, glass, plastic films, or fabrics.

The porous structure of nitrocellulose has excellent absorption and adsorption qualities for a wide variety of reagents including monoclonal antibodies. Nylon also possesses similar characteristics and also is suitable. It is contemplated that such porous solid supports described hereinabove are preferably in the form of sheets of thickness from about 0.01 to 0.5 mm, preferably about 0.1 mm. The pore size may vary within wide limits, and is preferably from about 0.025 to 15 microns, especially from about 0.15 to 15 microns. The surfaces of such supports may be activated by chemical processes which cause covalent linkage of the antigen or antibody to the support. The irreversible binding of the antigen or antibody is obtained, however, in general, by adsorption on the porous material by poorly understood hydrophobic forces. Suitable solid supports also are described in U.S. patent application Ser. No. 227,272.

The term "test sample" includes biological samples which can be tested by the methods of the present invention described herein and include human and animal body fluids such as whole blood, serum, plasma, cerebrospinal fluid, urine, lymph fluids, and various external secretions of the respiratory, intestinal and genitorurinary tracts, tears, saliva, milk, white blood cells, myelomas and the like, biological fluids such as cell culture supernatants, fixed tissue specimens and fixed cell specimens. Any substance which can be adapted for testing with the recombinant proteins described herein and assay formats of the present invention are contemplated to be within the scope of the present invention.

Other embodiments which utilize various other solid phases also are contemplated and are within the scope of this invention. For example, ion capture procedures for immobilizing an immobilizable reaction complex with a negatively charged polymer, described in co-pending U.S. patent application Ser. No. 150,278 corresponding to EP publication 0326100, and U.S. patent application Ser. No. 375,029 (EP publication no. 0406473) both of which enjoy common ownership and are incorporated herein by reference, can be employed according to the present invention to effect a fast solution-phase immunochemical reaction. An immobilizable immune complex is separated from the rest of the reaction mixture by ionic interactions between the negatively charged poly-anion/immune complex and the previously treated, positively charged porous matrix and detected by using various signal generating systems previously described, including those described in chemiluminescent signal measurements as described in co-pending U.S. patent application Ser. No. 921,979 corresponding to EPO Publication No. 0 273,115, which enjoys common ownership and which is incorporated herein by reference.

Also, the methods of the present invention can be adapted for use in systems which utilize microparticle technology including in automated and semi-automated systems wherein the solid phase comprises a microparticle. Such systems include those described in pending U.S. patent applications Ser. No. 425,651 and 425,643, which correspond to published EPO applications Nos. EP 0 425 633 and EP 0 424 634, respectively, which are incorporated herein by reference.

The use of scanning probe microscopy (SPM) for immunoassays also is a technology to which the recombinant proteins of the present invention or monoclonal antibodies produced from these recombinant proteins are easily adaptable. In scanning probe microscopy, in particular in atomic force microscopy, the capture phase, for example, either a recombinant protein produced by the novel plasmid described herein or a monoclonal antibody produced therefrom, is adhered to a solid phase, the test sample is contacted to the solid phase for a time and under conditions sufficient for a reaction between

the two to occur, and a scanning probe microscope is utilized to detect antigen/antibody complexes which may be present on the surface of the solid phase. The use of scanning tunnelling microscopy eliminates the need for labels which normally must be utilized in many immunoassay systems to detect antigen/antibody complexes.

In an assay format to detect the presence of antibody against a specific analyte (for example, an infectious agent such as a virus) in a human test sample, the human test sample is contacted and incubated with a solid phase coated with at least one recombinant protein (polypeptide). If antibodies are present in the test sample, they will form a complex with the antigenic polypeptide and become affixed to the solid phase. After the complex has formed, unbound materials and reagents are removed by washing the solid phase. The complex is reacted with an indicator reagent and allowed to incubate for a time and under conditions for second complexes to form. The presence of antibody in the test sample to the recombinant polypeptide(s) is determined by detecting the measurable signal generated. Signal generated above a cut-off value is indicative of antibody to the analyte present in the test sample. With many indicator reagents, such as enzymes, the amount of antibody present is proportional to the signal generated. Depending upon the type of test sample, it may be diluted with a suitable buffer reagent, concentrated, or contacted with the solid phase without any manipulation ("neat"). For example, it usually is preferred to test serum or plasma samples which previously have been diluted, or concentrate specimens such as urine, in order to determine the presence and/or amount of antibody present.

A sandwich assay is provided in still another embodiment. This method comprises contacting a test sample with a solid phase to which at least one recombinant antigen provided herein or a combination including at least one recombinant antigen provided herein are bound, to form a mixture. This mixture is incubated for a time and under conditions sufficient to allow antigen/antibody complexes to form. These complexes then are contacted with an indicator reagent comprising antigen(s) previously conjugated to a signal generating compound, to form a second mixture. This second mixture is incubated for a time and under conditions sufficient for antigen/antibody/indicator reagent complexes to form. The presence of the antigen/antibody/indicator reagent complexes is determined by detecting the measurable signal generated. In this assay, a first antigen which can be a recombinant antigen provided herein specific to the antibody to be detected is immobilized on a solid phase, a test sample suspected of containing the antibody is added to the solid phase, and a second antigen which can be a recombinant antigen of the invention having a label affixed thereto then is contacted with the solid phase. Thus, two recombinant antigens which are specific to a single binding pair member are used in one assay as a capture phase and a part of the indicator reagent. These antigens are the same and may be made in different, e.g., heterologous, sources. These sources could be bacterial and yeast, for example. It also is within the scope of the present invention that one recombinant antigen provided herein could be used as the capture reagent or as part of the indicator reagent, and the other antigen in this assay could be a synthetic peptide, or viral lysate, or obtained from other antigenic sources known to the routineer. Further, the use of biotin and antibiotin, biotin and avidin, biotin and streptavidin, and the like, may be used to enhance the generated signal in such assays.

In addition, more than one recombinant protein can be used in the assay format just described to test for the presence of antibody against a specific infectious agent by utilizing fusion proteins prepared as described herein against various antigenic epitopes of the viral genome of the infectious agent under study. Thus, it may be preferred to use recombinant polypeptides which contain epitopes within a specific viral antigenic region as well as epitopes from other antigenic regions from the viral genome to provide assays which have increased sensitivity any perhaps greater specificity than using a polypeptide from one epitope. Such an assay can be utilized as a confirmatory assay. In this particular assay format, a known amount of test sample is contacted with (a) known amount(s) of at least one solid support coated with at least one recombinant protein for a time and under conditions sufficient to form recombinant protein/antibody complexes. The complexes are contacted with known amount(s) of appropriate indicator reagent(s)s for a time and under suitable conditions for a reaction to occur, wherein the resultant signal generated is compared to a negative test sample in order to determine the presence of antibody to the analyte in the test sample. It further is contemplated that, when using certain solid phases such as microparticles, each recombinant protein utilized in the assay can be attached to a separate microparticle, and a mixture of these microparticles made by combining the various coated microparticles, which can be optimized for each assay.

Variations to the above-described assay formats include the incorporation of recombinant proteins produced by the plasmid descibed herein of different analytes attached to the same or to different solid phases for the detection of the presence of antibody to either analyte (for example, recombinant proteins specific for certain antigenic regions of HIV-1 coated on the same or different solid phase with recombinant proteins specific for certain antigenic region(s) of HIV-2, to detect the presence of either (or both) HIV-1 or HIV-2).

In yet another assay format, recombinant proteins produced from the plasmid described herein containing antigenic epitopes are useful in competitive assays such as neutralization assays. To perform a neutralization assay, a recombinant polypeptide representing epitopes of an antigenic region of an infectious agent such as a virus, is solubilized and mixed with a sample diluent to a final concentration of between 0.5 to 50.0 .mu.g/ml. A known amount of test sample (preferably 10 .mu.l), either diluted or non-diluted, is added to a reaction well, followed by 400 .mu.l of the sample diluent containing the recombinant polypeptide. If desired, the mixture may be preincubated for approximately 15 minutes to two hours. A solid phase coated with the recombinant protein described herein then is added to the reaction well, and incubated for one hour at approximately 40.degree. C. After washing, a known amount of an indicator reagent, for example, 200 .mu.l of a peroxide labelled goat anti-human IgG in a conjugate diluent is added and incubated for one hour at 40.degree. C. After washing and when using an enzyme conjugate such as described, an enzyme substrate, for example, OPD substrate, is added and incubated at room temperature for thirty minutes. The reaction is terminated by adding a stopping reagent such as 1N sulfuric acid to the reaction well. Absorbance is read at 492 nm. Test samples which contain antibody to the specific polypeptide generate a reduced signal caused by the competitive binding of the peptides to these antibodies in solution. The percentage of competitive binding may be calculated by comparing absorbance value of the sample in the presence of recombinant polypeptide to the absorbance value of the sample assayed in the absence of a recombinant polypeptide at the same dilution. Thus, the difference in the signals generated between the sample in the presence of recombinant protein and the sample in the absence of recombinant protein is the measurement used to determine the presence or absence of antibody.

Other neutralization assays are contemplated. These include competititve assays to detect the amount, if any, of an antigen analyte in a test sample. The assay comprises the steps of contacting a test sample with a known amount of analyte (in this instance, the recombinant antigen of the invention) having attached to it a signal generating compound and a solid phase to which has been attached an anti-analyte antibody. This resultant mixture is incubated for a time and under conditions sufficient to form either solid phaselanalyte complexes or solid phase/recombinant antigen complexes. The signal is triggered through means known in the art. Test samples which contain antibody to the specific polypeptide generate a reduced signal caused by the competitive binding of the antibody on the solid phase to the antigens in solution. The percentage of competitive binding may be calculated by comparing absorbance value of the sample in the presence of recombinant polypeptide to the absorbance value of the sample assayed in the absence of a recombinant polypeptide at the same dilution. Thus, the difference in the signals generated between the sample in the presence of recombinant protein and the sample in the absence of recombinant protein is the measurement used to determine the presence or absence of antibody.

In another assay format, the recombinant proteins can be used in immunodot blot assay systems. The immunodot blot assay system uses a panel of purified recombinant polypeptides placed in an array on a nitrocellulose solid support. The prepared solid support is contacted with a sample and captures specific antibodies (specific binding member) to the recombinant protein (other specific binding member) to form specific binding member pairs. The captured antibodies are detected by reaction with an indicator reagent. Preferably, the conjugate specific reaction is quantified using a reflectance optics assembly within an instrument which has been described in U.S. patent application Ser. No. 07/227,408 filed Aug. 2, 1988. The related U.S. patent application Ser. No. 07/227,586 and 07/227.590 (both of which were filed on Aug. 2, 1988) further described specific methods and apparatus useful to perform an immunodot assay, as well as U.S. Pat. No. 5,075,077 (U.S. Ser. No. 07/227,272 filed Aug. 2, 1988). Briefly, a nitrocellulose-base test cartridge is treated with multiple antigenic polypeptides. Each polypeptide is contained within a specific reaction zone on the test cartridge. After all the antigenic polypeptides have been placed on the nitrocellulose, excess binding sites on the nitrocellulose are blocked. The test cartridge then is contacted with a test sample such that each antigenic polypeptide in each reaction zone

will react if the test sample contains the appropriate antibody. After reaction, the test cartridge is washed and any antigen-antibody reactions are identified using suitable well-known reagents. As described in the patents and patent applications listed herein, the entire process is amenable to automation. The specifications of these applications related to the method and apparatus for performing an immunodot blot assay are incorporated herein by reference.

It also is within the scope of the present invention that fusion proteins prepared from the plasmid described herein can be used in assays which employ a first and second solid support, as follows, for detecting antibody to a specific antigen of an analyte in a test sample. In this assay format, a first aliquot of a test sample is contacted with a first solid support coated with recombinant protein specific for an analyte for a time and under conditions sufficient to form recombinant protein/analyte antibody complexes. Then, the complexes are contacted with an indicator reagent specific for the recombinant antigen. The indicator reagent is detected to determine the presence of antibody to the recombinant protein in the test sample. Following this, the presence of a different antigenic determinant of the same analyte is determined by contacting a second aliquot of a test sample with a second solid support coated with recombinant protein specific for the second antibody for a time and under conditions sufficient to form recombinant protein/second antibody complexes. The complexes are contacted with a second indicator reagent specific for the antibody of the complex. The signal is detected in order to determine the presence of antibody in the test sample, wherein the presence of antibody to either analyte recombinant protein, or both, indicates the presence of anti-analyte in the test sample. It also is contemplated that the solid supports can be tested simultaneously.

The use of haptens is known in the art. It is contemplated that haptens also can be used in assays employing fusion proteins produced by the plasmid of the invention in order to enhance performance of the assay.

The following examples are meant to illustrate, but not to limit, the spirit and scope of the invention.

EXAMPLES

Example 1

E2 Antigen Construction

Plasmid 577 was constructed by inserting coding sequence for a secretable HCV E2 antigen in two steps, as follows. First, a duplex synthetic oligonucleotide that had been digested with Spe 1 and Xba 1 was inserted into the Xba 1 cloning site of a previously constructed expression vector by sticky end ligation. This oligonucleotide contained sequence derived from rabbit immunoglobulin gamma chain peptide and other sequences included to create restriction sites for cloning purposes. It was then inserted downstream of a promoter element and RNA transcription start site. This DNA segment encoded amino acid sequences to be fused in frame at the Xba 1 sites to downstream gene sequences intended to be secreted from mammalian cells. The constructionof this plasmid 577 is shown in FIG. 1. The sequence of this DNA segment is depicted in FIG. 2. A conceptual translation delineating the mammalian secretion signal peptidase cleavage site is shown in FIG. 3.

Second, a PCR product containing sequence derived from an HCV plasmid template was inserted as an Xba 1 fragment downstream of the rabbit heavy chain signal sequence. Encoded in the "upper" PCR primer sequence was an Xba 1 site, immediately followed by 12 nucleotide sequence that encoded the amino acid sequence Serine-Asparagine-Glutamic Acid-Leucine ("SNEL") the amino terminal sequence of human pro-urokinase. The amino acid sequence SNEL was intended to promote signal protease processing, efficient secretion and final product stability in culture fluids. This segment is underlined in FIG. 2. Immediately following this 12 nucleotide sequence the primer contained nucleotides complementary to template sequences encoding amino acids starting at aa 388 of HCV. The "lower" PCR primer contained sequences homologous to template sequences that encode amino acids ending at 664 of HCV, a duplicate stop codon, and an Xba 1 site for cloning purposes. E2 antigen was truncated at this position to promote secretion. The Xba 1 sites appear in large bold type face and the stop codons are underlined in FIG. 2.

The complete sequence of this inserted region is depicted in FIG. 2 and in SEQUENCE I.D. NO. 1. A graphical representation of this coding region is depicted in FIG. 1.

Referring to FIG. 1, plasmid 577 contains the following DNA segments described counterlockwise from the top of the plasmid. FIG. 1: (a) a 2.3 Kb fragment of pBR322 containing bacterial beta-lactamase and origin of DNA replication; (b) a 1.8 Kb cassette directing expression of a neomycin resistance gene under control of HSV-1 thyridine kinase promoter and poly-A addition signals; (c) a 1.9 Kb cassette directing expression of a dihydrofolate reductase gene under the control of an SV-40 promoter and poly-A addition signals; (d) a 3.5 Kb cassette directing expression of a modified hepatitis C virus derived gene under the control of the Simian Virus 40 T-ag promoter and transcription enhancer, the hepatitis B virus surface antigen enhancer I, and a fragment of Herpes Simplex Virus-1 genome providing poly-A addition signals; and (e) a residual 0.7 Kb fragment of Simian Virus 40 genome late region of no function of this plasmid. These data are summarized in TABLE 1 hereinabove. All of the segments of the vector were assembled by standard methods know to those skilled in the art of molecular biology.

Example 2

A. Transfection of Dihydrofolate Reductase Deficient Chinese Hamster Ovary Cells.

The plasmid 577 was transfected into CHO/dhfr cells (dxb-111) (Uriacio, et al., Proc. Nat. Acad. Sci. 77, 4451-4466 (1980); these cells are available from the American Type Culture Collection [A.T.C.C.], 12301 Parklawn Drive, Rockville, Md. 20852, under Accession No. CRL 9096), using the cationic liposome-mediated procedure (Felgner, P. L. et al., Proc. Natl. Acad. Sci. 84, 7413-7417 (1987), as follows. CHO/dhfr cells were cultured in Ham's F-12 media supplemented with 10% fetal calf serum, L-glutamine (1 .mu.M) and freshly seeded into a 25 cm.sup.2 flask at a density of 5-8.times.10.sup.5 cells per flask twenty four hours prior to transfection. Fifteen micrograms of plasmid DNA was added to 1.5 mls of Opti-MEM I medium and 100 microliters of Lipofectin Reagent (Gibco-BRL, Grand Island N.Y.) was added to a second 1.5 ml portion of Opti-MEM I media. The two solutions were mixed and incubated at room temperature for 20 minutes. The culture medium was removed from cells and replaced with the Opti-MEM I-Lipofection-DNA solution for liposome-mediated transfection of DNA into cells in tissue culture. The cells were incubated for three hours at 37.degree. C. after which the Opti-MEM I-Lipofectin-DNA solution was replaced with culture medium for an additional 24 hours prior to selection.

B. Selection and Amplification.

One day after transfection, cells were passaged 1:3 and incubated with dhfr/G418 selection medium (hereafter, "F-12 minus medium G"). Selection medium was Ham's F-12 with L-glutamine and without hypoxanthine, thymidine, and glycine (JRH Biosciences, Lenexa, Kans., USA) and 300 micrograms per ml G418 (Gibco-BRL).

Colonies showing the presence of dihydrofolate reductase (Ringold, et al., J. Mol Appl. Genet. 1:165-174 (1981) plus aminoglycoside phosphotransferase (Southern, P. J. and Berg, P. J., Mol. Appl. Genet. 1:327-341 (1981) appeared after 4-5 days of incubation of transfected cells with F-12 minus medium G. After approximately two weeks, DHFR/G418 cells were sufficiently expanded to allow passage and continuous maintenance in F-12 minus medium G.

Amplification of the transfected UK-HCV-E2 gene was achieved by stepwise selection of DHFR.sup.+, G418.sup.+ cells with methotrexate (reviewed by Schimke, R., Cell 37, 705-713 (1984). Cells were incubated with F-12 minus medium G, containing 150 nM methotrexate (MTX), for approximately two weeks until resistant colonies appeared. The MTX resistant cells were passaged and maintained in the appropriate selection medium. Further amplification was achieved by selection with 5 .mu.M MTX, and cells continuously maintained in the appropriate selection medium.

C. Maintenance and Storage of Cell Lines.

Cells in culture and undergoing various selection or amplification procedures were re-fed with the

appropriate culture medium three times weekly. Cells were passaged 1:4, with appropriate medium, into 75 cm.sup.2 flasks and incubated at 37.degree. C. with 5% CO.sub.2 using standard methods. Cryostorage was by resuspension of 2-4.times.10.sup.6 cells in 1.8 ml of the appropriate culture medium containing 5% DMSO (Sigma Chem. Co., St. Louis, Mo., USA) and cold storage for 24 hours at -80.degree. C. and then permanent storage at -135.degree. C.

D. Antigen Production.

Ham's F12 custom minus medium was overlayed onto just confluent monolayers for 12 to 24 hours at 37.degree. C. in 5% CO.sub.2. Then, the growth medium was removed and the cells were rinsed three times with phosphate buffered saline (PBS) (with calcium and magnesium) available from Gibco-BRL, to remove the remaining media/serum which might be present. Cells then were incubated with VAS custom medium (VAS custom formulation with 1-glutamine with HEPES without phenol red, available from JRH Bioscience, product number 52-08678P), for one hour at 37.degree. C. in 5% CO.sub.2. As a final wash, the VAS then was discarded. Cells then were overlaid with VAS for production at 5 mls per T 25 cm.sup.2 flask, scaled proportionally for larger flasks or roller bottles). For harvest 1, the medium is removed after three to four days of incubation and then frozen to await purification with harvests 2 and 3. The monolayers were overlayed with VAS for two more three to four day harvests. The cultures were observed daily to determine cell conditions.

E. Clarification and Concentration.

Harvests were clarified at 1500.times.g for 30 minutes. Supernatants were concentrated to 50.times. in an Amicon stirred cell equipped with an Amicon YM10 membrane (available from Amicon, Amicon, Beverly, Mass.).

Example 3

CHO-E2 Purification

The sialic acid containing CHO-E2 glycoprotein was purified to greater than 90% purity from cell supernatants by ion exchange and lectin chromatography. Ten (10) separate lots from two different protein-free media all were purified, which demonstrated the reproducibility and versatility of this procedure. Purity was evaluated by R-250 coomassie and silver staining. Theoretical molecular weight of 30 Kdal was verified by Endo-F digestion.

Supernatants from cells propagated in roller bottles were spun to remove cell debris and then concentrated using an Amicon YM10 membrane to 50.times. (50 ml). The 50.times. concentrate was final filtered through a 0.2 .mu.m filter and then extensively dialyzed (12-14 Kdal cut-off) against S-Sepharose running buffer (0.02 M sodium phosphate, no salt, pH 6.5). The ion-exchange chromatography consisted of two columns (S-Sepharose and DEAE-Sepharose). Both columns were run in series, unwanted proteins were bound onto the columns while the protein of interest was contained in the flow. The ion exchange columns were cleaned to remove the unwanted proteins with 2 M NaCl in the columns' respective running buffer.

The concentrated and dialyzed supernatant first was loaded on an equilibrated S-Sepharose column (200 ml bed volume) at a flow rate of 5 ml/min. The unbound flow was collected, concentrated (YM10) to original volume and extensively dialyzed in DEAE-Sepharose running buffer (containing 0.02 M Tris buffer/0.1 M NaCl, pH 8.5). It was found that the conductivity of this buffer should be about 12 mS. After dialysis, the material was loaded onto a 200 ml DEAE-Sepharose column at a flow rate of 5 ml/min. The unbound flow was collected, concentrated (YM10) to original volume and extensively dialyzed in 0.01 M sodium phosphate, 0.13 M NaCl, pH 7.0. This buffer was termed the lectin WGA-Sepharose 6MB running buffer. Once the sample was changed into WGA running buffer, it was loaded at 0.5 ml/min onto a 10.0 ml WGA-Sepharose 6 MB column, collecting and recirculating the flow. After extensive washing (10 column volumes), the column flow was reversed and the purified CHO-E2 antigen was eluted using 10 mM N,N'-diacetylchitobiose in running buffer. The purified antigen was dialyzed against PBS and stored at -70.degree. C.

Example 4

CHO-E2 Antigen Assay for Screening Cloned Cell Suspensions

A. Preparation of CHO-E2 Antigen Beads. Twenty microliters (20 .mu.l) of cloned cell suspension or control suspensions (CHO cells transfected with expression vector containing no HCV insert) were placed into a microtiter well which was capable of containing a 1/4 inch bead. The number of wells being tested was multiplied by 0.2 and by 1.05 to obtain the volume in milliliters (ml) of diluent necessary for coating. The diluent used was SMP diluent (available from Abbott Laboratories, Abbott Park, Ill.).

Two hundred microliters (200 .mu.l) of the reagent obtained hereinabove was added to each well containing supernatant, sealed, and the tray containing the wells was placed in an incubator pre-warmed to 40.degree. C. The trays were shaken in the Dynamic Incubator on Dynamic mode for 20 seconds in order to mix the samples. Following this, the trays were incubated for one hour at 40.degree. C. in a static state. Then, the E2 peptide bead was added, covered and incubated for one hour at 40.degree. C. in a static mode. Following this incubation, the trays were washed and 200 .mu.l of conjugate was added per well (100 ng/ml of gamma spec G anti-human HRPO in HCV 2.0 conjugate diluent, list number 4A14C, available from Abbott Laboratories, Abbott Park, Ill.). This mixture was incubated at 40.degree. C. in a static mode for 30 minutes. Following this incubation, the beads were washed and then transferred to an EIA tube box. Then, 300 .mu.l of OPD substrate (available from bbott Laboratories, Abbott Park, Ill.) was added to each well and the resulting ixture was incubated for 30 minutes at 40.degree. C. The reaction was stopped by adding 1.0 ml of 1 N H.sub.2 SO.sub.4 per well. Each well was read at an absorbance of 492 (A.sub.492) on an Abbott Quantum.TM. instrument, and the validity of the assay then was determined as follows:

Average A.sub.492 of the negative control =1.000+/-0.10; the average A.sub.492 of the positive control =0.025+/-0.10. Following these calculations, the percent reduction of the test sample was calculated as follows: (Average A.sub.492 sample)+1.times.100=% Reduction

Average A.sub.492 Neg Control

Example 5

Assays Utilizing E2 Antigen

Purified HCV E2 antigen prepared as described in the previous examples was coated onto polystyrene beads following methods well-known in the art at a concentration of 1.0 to 2.0 .mu.g/ml. The components used in the coating procedure were adjusted to provide optimum sensitivity and specificity for the antibody assay test. The specificity of the E2 antibody assay was evaluated by testing specimens from populations of volunteer blood donors. All specimens were testd at 1:41 dilution in the enzyme immunoassay using goat anti-human IgG labeled with horseradish peroxidase as the signal generating compound according to the assay protocol hereinabove (see previous examples).

Chronic and acute non-A, non-B hepatitis (NANBH) specimens were obtained from multiple U.S. sites. Serially collected specimens from individuals seroconverting to HCV antigens were obtained from commercial plasma vendors. Archived samples which were HCV RNA positive (N=495) were obtained from a large virology reference laboratory in the U.S. without linkage to patients or donors. The RNA extraction and PCR amplification procedures have been described (D. Gretch et al., J. Clin. Micro. 30:2145-2149 (1992). Additional HCV RNA positive specimens were collected from Japan (C=59) and the Netherlands (N=33). Specimens from blood donors at risk for HCV infection (N=304) with ALT values greater than 100 IU/L were obtained from the New York Blood Center. Samples indeterminately reactive on Abbott MATRIX.TM. 1.0 HCV assay for HCV core antigen (N=139) and HCV NS3 antigen (N=149) were obtained from the Abbott Virology Reference Laboratory, North Chicago, Ill. A commerically available anti-HCV 2.0 positive blood donors and patients. A commerically available anti-HCV mixed titer panel (PHV 203) was obtained from Boston Biomedica, Inc. (BBI), West Bridgewater, MA. HCV 2.0 reactive plasma samples were obtained from North American Biologicals, Inc. (NABI) and only samples which were concordantly reactive in two HCV 2.0 EIA (available from

Abbott Laboratories, Abbott Park, Ill. and Ortho Diagnostics, Inc., Raritan, N.J.) were analyzed further.

One hundred fifity nine (159) patients previously diagnosed with chronic NANBH were tested using the Abbott HCV 2.0 test and the E2 EIA. A total of 147/159 (92.5%) patients were positive with HCV 2.0 while 141/159 (88.5%) patients also had antibody to E2. Overall, there was 96.2% agreement between the HCV 2.0 and E2 assays. A high correlation (94%) between HCV core and E2 antibodies also was observed in this population. A similar high concordance was seen between HCV 2.0 and E2 assays in acute NANBH patients. Ninety-nine (99) of these 113 (87.6%) specimens gave concordant results (51 positive and 48 negative), while 10 specimens reacted exclusively with HCV 2.0 and 4 specimens were positive only in the E2 antibody assay. The overall reactive rates in acute patients for HCV 2.0 and the E2 EIA were 54% and 49%, respectively.

Serially collected specimens from 5 individual plasma donors who seroconverted to multiple HCV antigens were also shown to react wit the HCV E2 protein. In three of the five patients, E2 antibody was the first antibody detectable during seroconversion. Anti-E2 eventually appeared in all five cases.

A total of 587 individuals HCV RNA positive specimens were tested for antibody to E2 as well as for other individual HCV antibodies using the Abbott MATRIX.TM. HCV 2.0 assay Five hundred seventy-one (571) of 587 of these RNA positive specimens werc shown to contain antibodies to E2, including 56/59 (94.9%) of the specimens collected in Japan. All E2 positive samples contained other HCV antibodies as detected by Abbott MATRIX.TM., but no single antibody occurred with greater frequency than E2 antibody in this population.

Among the cohort of blood donors with ALT values greater than 100 IU/L, 48 (15.8%) were positive for E2 antibodies. Forty-six (95.8%) of these 48 donors also were reactive in the Abbott HCV 2.0 EIA and were confirmed reactive in the Abbott MATRIX.TM. HCV 2.0 assay.

Specimens detected by Abbott MATRIX.TM. HCV 1.0 as having antibodies to HCV core or HCV NS3 exclusively, were tested for E2 antibodies. Fifty-nine (59) (42.4%) of 139 core reactive specimens were found to contain E2 antibodies as were 23/149 (15.4%) NS3 reactives.

A panel of well-characterized specimens with regard to serological markers was obtained from BBI and evaluated with the Abbott HCV 3.0 EIA and E2 antibody tests. HCV 2.0 EIA (Abbott and Ortho), HCV 3.0 EIA (Ortho), Abbott MATRIX.TM. HCV 1.0 and RIBA HCV 2.0 data were supplied by BBI with the panel. Eighteen (18) of 23 (78.3%) HCV 2.0 EIA positive (by both Abbott and Ortho assays) specimens were also anti-E2 positive. The two HCV negative panel members were E2 antibody negative.

Among the 23 HCV 2.0 concordantly positive specimens were six samples (26.1% of total) which scored negative in the Ortho HCV 3.0 EIA but remained reactive in the Abbott HCV 3.0 EIA. Three 50% of these six specimens were shown to contain antibodies to multiple HCV proteins including 2 specimens which had antibodies to E2. These two specimens were reactive to HCV core in both RIBA and Abbott MATRIX.TM. assays.

Thus, the E2 recombinant antigen from plasmid 577 was able to function in assays which employed it.

Other modifications and variations of the specific embodiments of the invention as set forth herein will be apparent to those skilled in the art. Accordingly, the invention is intended to be limited in accordance with the appended claims.

```
#                SEQUENCE LISTING
- (1) GENERAL INFORMATION:
-    (iii) NUMBER OF SEQUENCES: 2
- (2) INFORMATION FOR SEQ ID NO:1:
-      (i) SEQUENCE CHARACTERISTICS:
#pairs    (A) LENGTH: 937 base
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: double
```

```
          (D) TOPOLOGY: linear
-     (ii) MOLECULE TYPE: DNA (genomic)
-     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- ACTAGTCGAC ATGGAGACTG GGCTGCGCTG GCTTCTCCTG GTCGCTGTGC TC - #AAAGGTGT
  60
- CCAGTGTCTC GAGGGGTCTA GAAGCAATGA ACTTACCGGG GGAAGTGCCG GC - #CACACCAC
 120
- GGCTGGGCTT GTTCGTCTCC TTTCACCAGG CGCCAAGCAG AACATCCAAC TG - #ATCAACAC
 180
- CAACGGCAGT TGGCACATCA ATAGCACGGC CTTGAACTGC AATGAAAGCC TT - #AACACCGG
 240
- CTGGTTAGCA GGGCTCTTCT ATCACCACAA ATTCAACTCT TCAGGTTGTC CT - #GAGAGGTT
 300
- GGCCAGCTGC CGACGCCTTA CCGATTTTGC CCAGGGCGGG GGTCCTATCA GT - #TACGCCAA
 360
- CGGAAGCGGC CTCGATGAAC GCCCCTACTG CTGGCACTAC CCTCCAAGAC CT - #TGTGGCAT
 420
- TGTGCCCGCA AAGAGCGTGT GTGGCCCGGT ATATTGCTTC ACTCCCAGCC CC - #GTGGTGGT
 480
- GGGAACGACC GACAGGTCGG GCGCGCCTAC CTACAGCTGG GGTGCAAATG AT - #ACGGATGT
 540
- CTTTGTCCTT AACAACACCA GGCCACCGCT GGGCAATTGG TTCGGTTGCA CC - #TGGATGAA
 600
- CTCAACTGGA TTCACCAAAG TGTGCGGAGC GCCCCCTTGT GTCATCGGAG GG - #GTGGGCAA
 660
- CAACACCTTG CTCTGCCCCA CTGATTGCTT CCGCAAGCAT CCGGAAGCCA CA - #TACTCTCG
 720
- GTGCGGCTCC GGTCCCTGGA TTACACCCAG GTGCATGGTC GACTACCCGT AT - #AGGCTTTG
 780
- GCACTATCCT TGTACCATCA ATTACACCAT ATTCAAAGTC AGGATGTACG TG - #GGAGGGGT
 840
- CGAGCACAGG CTGGAAGCGG CCTGCAACTG GACGCGGGGC GAACGCTGTG AT - #CTGGAAGA
 900
#     937           CTCA GCCCGTGATA ATCTAGA
- (2) INFORMATION FOR SEQ ID NO:2:
-      (i) SEQUENCE CHARACTERISTICS:
#acids    (A) LENGTH: 305 amino
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
-     (ii) MOLECULE TYPE: protein
-     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
- Met Glu Thr Gly Leu Arg Trp Leu Leu Leu Va - #l Ala Val Leu Lys Gly
#                15
- Val Gln Cys Leu Glu Gly Ser Arg Ser Asn Gl - #u Leu Thr Gly Gly Ser
#            30
- Ala Gly His Thr Thr Ala Gly Leu Val Arg Le - #u Leu Ser Pro Gly Ala
#        45
- Lys Gln Asn Ile Gln Leu Ile Asn Thr Asn Gl - #y Ser Trp His Ile Asn
#    60
- Ser Thr Ala Leu Asn Cys Asn Glu Ser Leu As - #n Thr Gly Trp Leu Ala
#80
- Gly Leu Phe Tyr His His Lys Phe Asn Ser Se - #r Gly Cys Pro Glu Arg
#                95
- Leu Ala Ser Cys Arg Arg Leu Thr Asp Phe Al - #a Gln Gly Gly Gly Pro
#            110
- Ile Ser Tyr Ala Asn Gly Ser Gly Leu Asp Gl - #u Arg Pro Tyr Cys Trp
#        125
- His Tyr Pro Pro Arg Pro Cys Gly Ile Val Pr - #o Ala Lys Ser Val Cys
#   140
- Gly Pro Val Tyr Cys Phe Thr Pro Ser Pro Va - #l Val Val Gly Thr Thr
145                 1 - #50                 1 - #55                 1 -
#60
- Asp Arg Ser Gly Ala Pro Thr Tyr Ser Trp Gl - #y Ala Asn Asp Thr Asp
#                175
- Val Phe Val Leu Asn Asn Thr Arg Pro Pro Le - #u Gly Asn Trp Phe Gly
#            190
- Cys Thr Trp Met Asn Ser Thr Gly Phe Thr Ly - #s Val Cys Gly Ala Pro
#        205
```

```
- Pro Cys Val Ile Gly Gly Val Gly Asn Asn Th - #r Leu Leu Cys Pro Thr
#   220
- Asp Cys Phe Arg Lys His Pro Glu Ala Thr Ty - #r Ser Arg Cys Gly Ser
225                 2 - #30                 2 - #35                 2 -
#40
- Gly Pro Trp Ile Thr Pro Arg Cys Met Val As - #p Tyr Pro Tyr Arg Leu
#               255
- Trp His Tyr Pro Cys Thr Ile Asn Tyr Thr Il - #e Phe Lys Val Arg Met
#           270
- Tyr Val Gly Gly Val Glu His Arg Leu Glu Al - #a Ala Cys Asn Trp Thr
#       285
- Arg Gly Glu Arg Cys Asp Leu Glu Asp Arg As - #p Arg Ser Glu Leu Ser
#   300
- Pro
305
```

---

* * * * *
